RECOMBINANT METHODS AND MATERIALS FOR PRODUCING EPOTHILONE AND EPOTHILONE DERIVATIVES

INS A1

Cross-Reference to Related Applications

This application claims priority to U.S. provisional application Serial Nos. 60/130,560, filed 22 Apr. 1999; 60/122,620, filed 3 Mar. 1999; 60/119,386, filed 10 Feb. 1999; and 60/109,401, filed 20 Nov. 1998, each of which is incorporated herein by reference.

Reference to Government Funding

This invention was supported in part by SBIR grant 1R43-CA79228-01. The U.S. government has certain rights in this invention.

Field of the Invention

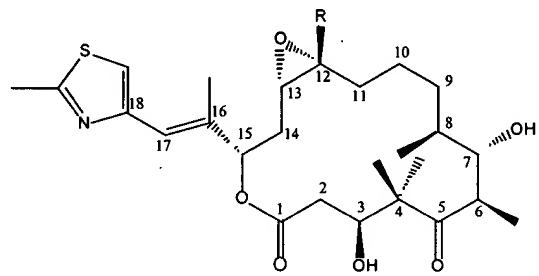
The present invention provides recombinant methods and materials for producing epothilone and epothilone derivatives. The invention relates to the fields of agriculture, chemistry, medicinal chemistry, medicine, molecular biology, and pharmacology.

Background of the Invention

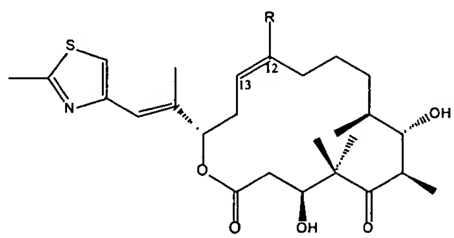
The epothilones were first identified by Gerhard Hofle and colleagues at the National Biotechnology Research Institute as an antifungal activity extracted from the myxobacterium *Sorangium cellulosum* (see K. Gerth *et al.*, 1996, J. Antibiotics 49: 560-563 and Germany Patent No. DE 41 38 042). The epothilones were later found to have activity in a tubulin polymerization assay (see D. Bollag *et al.*, 1995, Cancer Res. 55:2325-2333) to identify antitumor agents and have since been extensively studied as potential antitumor agents for the treatment of cancer.

The chemical structure of the epothilones produced by *Sorangium cellulosum* strain So ce 90 was described in Hofle *et al.*, 1996, Epothilone A and B - novel 16-membered macrolides with cytotoxic activity: isolation, crystal structure, and conformation in solution, Angew. Chem. Int. Ed. Engl. 35(13/14): 1567-1569,

incorporated herein by reference. The strain was found to produce two epothilone compounds, designated A (R = H) and B (R = CH_3), as shown below, which showed broad cytotoxic activity against eukaryotic cells and noticeable activity and selectivity against breast and colon tumor cell lines.



The desoxy counterparts of epothilones A and B, also known as epothilones C (R = H) and D (R = CH_3), are known to be less cytotoxic, and the structures of these epothilones are shown below.



Two other naturally occurring epothilones have been described. These are epothilones E and F, in which the methyl side chain of the thiazole moiety of epothilones A and B has been hydroxylated to yield epothilones E and F, respectively.

Because of the potential for use of the epothilones as anticancer agents, and because of the low levels of epothilone produced by the native So ce 90 strain, a number of research teams undertook the effort to synthesize the epothilones. This effort has been successful (see Balog *et al.*, 1996, Total synthesis of (-)-epothilone A, Angew. Chem. Int. Ed. Engl. 35(23/24): 2801-2803; Su *et al.*, 1997, Total synthesis of (-)-epothilone B: an extension of the Suzuki coupling method and insights into structure-activity relationships of the epothilones, Angew. Chem. Int. Ed. Engl. 36(7): 757-759; Meng *et al.*, 1997, Total syntheses of epothilones A and B, JACS 119(42): 10073-10092; and Balog *et al.*, 1998, A novel aldol condensation with 2-methyl-4-pentenal and its application to an improved total synthesis of epothilone B, Angew. Chem. Int. Ed. Engl. 37(19): 2675-2678, each of

which is incorporated herein by reference). Despite the success of these efforts, the chemical synthesis of the epothilones is tedious, time-consuming, and expensive. Indeed, the methods have been characterized as impractical for the full-scale pharmaceutical development of an epothilone.

A number of epothilone derivatives, as well as epothilones A - D, have been studied *in vitro* and *in vivo* (see Su *et al.*, 1997, Structure-activity relationships of the epothilones and the first *in vivo* comparison with paclitaxel, Angew. Chem. Int. Ed. Engl. 36(19): 2093-2096; and Chou *et al.*, Aug. 1998, Desoxyepothilone B: an efficacious microtubule-targeted antitumor agent with a promising *in vivo* profile relative to epothilone B, Proc. Natl. Acad. Sci. USA 95: 9642-9647, each of which is incorporated herein by reference). Additional epothilone derivatives and methods for synthesizing epothilones and epothilone derivatives are described in PCT patent publication Nos. 99/54330, 99/54319, 99/54318, 99/43653, 99/43320, 99/42602, 99/40047, 99/27890, 99/07692, 99/02514, 99/01124,98/25929, 98/22461, 98/08849, and 97/19086; U.S. Patent No. 5,969,145; and Germany patent publication No. DE 41 38 042, each of which is incorporated herein by reference.

There remains a need for economical means to produce not only the naturally occurring epothilones but also the derivatives or precursors thereof, as well as new epothilone derivatives with improved properties. There remains a need for a host cell that produces epothilones or epothilone derivatives that is easier to manipulate and ferment than the natural producer *Sorangium cellulosum*. The present invention meets these and other needs.

Summary of the Invention

In one embodiment, the present invention provides recombinant DNA compounds that encode the proteins required to produce epothilones A, B, C, and D. The present invention also provides recombinant DNA compounds that encode portions of these proteins. The present invention also provides recombinant DNA compounds that encode a hybrid protein, which hybrid protein includes all or a portion of a protein involved in epothilone biosynthesis and all or a portion of a protein involved in the biosynthesis of

another polyketide or non-ribosomal-derived peptide. In a preferred embodiment, the recombinant DNA compounds of the invention are recombinant DNA cloning vectors that facilitate manipulation of the coding sequences or recombinant DNA expression vectors that code for the expression of one or more of the proteins of the invention in recombinant host cells.

In another embodiment, the present invention provides recombinant host cells that produce a desired epothilone or epothilone derivative. In one embodiment, the invention provides host cells that produce one or more of the epothilones or epothilone derivatives at higher levels than produced in the naturally occurring organisms that produce epothilones. In another embodiment, the invention provides host cells that produce mixtures of epothilones that are less complex than the mixtures produced by naturally occurring host cells. In another embodiment, the present invention provides non-*Sorangium* recombinant host cells that produce an epothilone or epothilone derivative.

In a preferred embodiment, the host cells of the invention produce less complex mixtures of epothilones than do naturally occurring cells that produce epothilones. Naturally occurring cells that produce epothilones typically produce a mixture of epothilones A, B, C, D, E, and F. The table below summarizes the epothilones produced in different illustrative host cells of the invention.

Cell Type	Epothilones Produced	Epothilones Not Produced
1	A, B, C, D, E, F	-----
2	A, C, E	B, D, F
3	B, D, F	A, C, E
4	A, B, C, D	E, F
5	A, C	B, D, E, F
6	C	A, B, D, E, F
7	B, D	A, C, E, F
8	D	A, B, C, E, F

In addition, cell types may be constructed which produce only the newly discovered epothilones G and H, further discussed below, and one or the other of G and

H or both in combination with the downstream epothilones. Thus, it is understood, based on the present invention, that the biosynthetic pathway which relates the naturally occurring epothilones is, respectively, G → C → A → E and H → D → B → F. Appropriate enzymes may also convert members of each pathway to the corresponding member of the other.

Thus, the recombinant host cells of the invention also include host cells that produce only one desired epothilone or epothilone derivative.

In another embodiment, the invention provides *Sorangium* host cells that have been modified genetically to produce epothilones either at levels greater than those observed in naturally occurring host cells or as less complex mixtures of epothilones than produced by naturally occurring host cells, or produce an epothilone derivative that is not produced in nature. In a preferred embodiment, the host cell produces the epothilones at equal to or greater than 20 mg/L.

In another embodiment, the recombinant host cells of the invention are host cells other than *Sorangium cellulosum* that have been modified genetically to produce an epothilone or an epothilone derivative. In a preferred embodiment, the host cell produces the epothilones at equal to or greater than 20 mg/L. In a more preferred embodiment, the recombinant host cells are *Myxococcus*, *Pseudomonas*, or *Streptomyces* host cells that produce the epothilones or an epothilone derivative at equal to or greater than 20 mg/L. In another embodiment, the present invention provides novel compounds useful in agriculture, veterinary practice, and medicine. In one embodiment, the compounds are useful as fungicides. In another embodiment, the compounds are useful in cancer chemotherapy. In a preferred embodiment, the compound is an epothilone derivative that is at least as potent against tumor cells as epothilone B or D. In another embodiment, the compounds are useful as immunosuppressants. In another embodiment, the compounds are useful in the manufacture of another compound. In a preferred embodiment, the compounds are formulated in a mixture or solution for administration to a human or animal.

These and other embodiments of the invention are described in more detail in the following description, the examples, and claims set forth below.

Brief Description of the Figures

Figure 1 shows a restriction site map of the insert *Sorangium cellulosum* genomic DNA in four overlapping cosmid clones (designated 8A3, 1A2, 4, and 85 and corresponding to pKOS35-70.8A3, pKOS35-70.1A2, pKOS35-70.4, and pKOS35-79.85, respectively) spanning the epothilone gene cluster. A functional map of the epothilone gene cluster is also shown. The loading domain (Loading, *epoA*), the non-ribosomal peptide synthase (NRPS, Module 1, *epoB*) module, and each module (Modules 2 through 9, *epoC*, *epoD*, *epoE*, and *epoF*) of the remaining eight modules of the epothilone synthase gene are shown, as is the location of the *epoK* gene that encodes a cytochrome P450-like epoxidation enzyme.

Figure 2 shows a number of precursor compounds to N-acylcysteamine thioester derivatives that can be supplied to an epothilone PKS of the invention in which the NRPS-like module 1 or module 2 KS domain has been inactivated to produce a novel epothilone derivative. A general synthetic procedure for making such compounds is also shown.

Figure 3 shows restriction site and function maps of plasmids pKOS35-82.1 and pKOS35-82.2.

Figure 4 shows restriction site and function maps of plasmids pKOS35-154 and pKOS90-22.

Figure 5 shows a schematic of a protocol for introducing the epothilone PKS and modification enzyme genes into the chromosome of a *Myxococcus xanthus* host cell as described in Example 3.

Figure 6 shows restriction site and function maps of plasmids pKOS039-124 and pKOS039-124R.

Figure 7 shows a restriction site and function map of plasmid pKOS039-126R.

Figure 8 shows a restriction site and function map of plasmid pKOS039-141.

Figure 9 shows a restriction site and function map of plasmid pKOS045-12.

Detailed Description of the Invention

The present invention provides the genes and proteins that synthesize the epothilones in *Sorangium cellulosum* in recombinant and isolated form. As used herein, the term recombinant refers to a compound or composition produced by human intervention, typically by specific and directed manipulation of a gene or portion thereof. The term isolated refers to a compound or composition in a preparation that is substantially free of contaminating or undesired materials or, with respect to a compound or composition found in nature, substantially free of the materials with which that compound or composition is associated in its natural state. The epothilones (epothilone A, B, C, D, E, and F) and compounds structurally related thereto (epothilone derivatives) are potent cytotoxic agents specific for eukaryotic cells. These compounds have application as anti-fungals, cancer chemotherapeutics, and immunosuppressants. The epothilones are produced at very low levels in the naturally occurring *Sorangium cellulosum* cells in which they have been identified. Moreover, *S. cellulosum* is very slow growing, and fermentation of *S. cellulosum* strains is difficult and time-consuming. One important benefit conferred by the present invention is the ability simply to produce an epothilone or epothilone derivative in a non-*S. cellulosum* host cell. Another advantage of the present invention is the ability to produce the epothilones at higher levels and in greater amounts in the recombinant host cells provided by the invention than possible in the naturally occurring epothilone producer cells. Yet another advantage is the ability to produce an epothilone derivative in a recombinant host cell.

The isolation of recombinant DNA encoding the epothilone biosynthetic genes resulted from the probing of a genomic library of *Sorangium cellulosum* SMP44 DNA. As described more fully in Example 1 below, the library was prepared by partially digesting *S. cellulosum* genomic DNA with restriction enzyme SauIIIA1 and inserting the DNA fragments generated into BamHI-digested Supercos™ cosmid DNA (Stratagene). Cosmid clones containing epothilone gene sequences were identified by probing with DNA probes specific for sequences from PKS genes and reprobing with secondary probes comprising nucleotide sequences identified with the primary probes.

Four overlapping cosmid clones were identified by this effort. These four cosmids were deposited with the American Type Culture Collection (ATCC), Manassas, VA, USA, under the terms of the Budapest Treaty, and assigned ATCC accession numbers. The clones (and accession numbers) were designated as cosmids pKOS35-70.1A2 (ATCC 203782), pKOS35-70.4 (ATCC 203781), pKOS35-70.8A3 (ATCC 203783), and pKOS35-79.85 (ATCC 203780). The cosmids contain insert DNA that completely spans the epothilone gene cluster. A restriction site map of these cosmids is shown in Figure 1. Figure 1 also provides a function map of the epothilone gene cluster, showing the location of the six epothilone PKS genes and the *epoK* P450 epoxidase gene.

The epothilone PKS genes, like other PKS genes, are composed of coding sequences organized to encode a loading domain, a number of modules, and a thioesterase domain. As described more fully below, each of these domains and modules corresponds to a polypeptide with one or more specific functions. Generally, the loading domain is responsible for binding the first building block used to synthesize the polyketide and transferring it to the first module. The building blocks used to form complex polyketides are typically acylthioesters, most commonly acetyl, propionyl, malonyl, methylmalonyl, and ethylmalonyl CoA. Other building blocks include amino acid-like acylthioesters. PKSs catalyze the biosynthesis of polyketides through repeated, decarboxylative Claisen condensations between the acylthioester building blocks. Each module is responsible for binding a building block, performing one or more functions on that building block, and transferring the resulting compound to the next module. The next module, in turn, is responsible for attaching the next building block and transferring the growing compound to the next module until synthesis is complete. At that point, an enzymatic thioesterase (TE) activity cleaves the polyketide from the PKS.

Such modular organization is characteristic of the class of PKS enzymes that synthesize complex polyketides and is well known in the art. Recombinant methods for manipulating modular PKS genes are described in U.S. Patent Nos. 5,672,491; 5,712,146; 5,830,750; and 5,843,718; and in PCT patent publication Nos. 98/49315 and 97/02358, each of which is incorporated herein by reference. The polyketide known as 6-deoxyerythronolide B (6-dEB) is synthesized by a PKS that is a prototypical modular

PKS enzyme. The genes, known as *eryAI*, *eryAII*, and *eryAIII*, that code for the multi-subunit protein known as deoxyerythronolide B synthase or DEBS (each subunit is known as DEBS1, DEBS2, or DEBS3) that synthesizes 6-dEB are described in U.S. Patent Nos. 5,712,146 and 5,824,513, incorporated herein by reference.

The loading domain of the DEBS PKS consists of an acyltransferase (AT) and an acyl carrier protein (ACP). The AT of the DEBS loading domain recognizes propionyl CoA (other loading domain ATs can recognize other acyl-CoAs, such as acetyl, malonyl, methylmalonyl, or butyryl CoA) and transfers it as a thioester to the ACP of the loading domain. Concurrently, the AT on each of the six extender modules recognizes a methylmalonyl CoA (other extender module ATs can recognize other CoAs, such as malonyl or alpha-substituted malonyl CoAs, i.e., malonyl, ethylmalonyl, and 2-hydroxymalonyl CoA) and transfers it to the ACP of that module to form a thioester. Once DEBS is primed with acyl- and methylmalonyl-ACPs, the acyl group of the loading domain migrates to form a thioester (trans-esterification) at the KS of the first module; at this stage, module one possesses an acyl-KS adjacent to a methylmalonyl ACP. The acyl group derived from the DEBS loading domain is then covalently attached to the alpha-carbon of the extender group to form a carbon-carbon bond, driven by concomitant decarboxylation, and generating a new acyl-ACP that has a backbone two carbons longer than the loading unit (elongation or extension). The growing polyketide chain is transferred from the ACP to the KS of the next module of DEBS, and the process continues.

The polyketide chain, growing by two carbons for each module of DEBS, is sequentially passed as a covalently bound thioester from module to module, in an assembly line-like process. The carbon chain produced by this process alone would possess a ketone at every other carbon atom, producing a polyketone, from which the name polyketide arises. Commonly, however, additional enzymatic activities modify the beta keto group of each two carbon unit just after it has been added to the growing polyketide chain but before it is transferred to the next module. Thus, in addition to the minimal module containing KS, AT, and ACP necessary to form the carbon-carbon bond, modules may contain a ketoreductase (KR) that reduces the keto group to an alcohol.

Modules may also contain a KR plus a dehydratase (DH) that dehydrates the alcohol to a double bond. Modules may also contain a KR, a DH, and an enoylreductase (ER) that converts the double bond to a saturated single bond using the beta carbon as a methylene function. The DEBS modules include those with only a KR domain, only an inactive KR domain, and with all three KR, DH, and ER domains.

Once a polyketide chain traverses the final module of a PKS, it encounters the releasing domain or thioesterase found at the carboxyl end of most PKSs. Here, the polyketide is cleaved from the enzyme and, for most but not all polyketides, cyclized. The polyketide can be modified further by tailoring or modification enzymes; these enzymes add carbohydrate groups or methyl groups, or make other modifications, i.e., oxidation or reduction, on the polyketide core molecule. For example, 6-dEB is hydroxylated, methylated, and glycosylated (glycosidated) to yield the well known antibiotic erythromycin A in the *Saccharopolyspora erythraea* cells in which it is produced naturally.

While the above description applies generally to modular PKS enzymes and specifically to DEBS, there are a number of variations that exist in nature. For example, many PKS enzymes comprise loading domains that, unlike the loading domain of DEBS, comprise an "inactive" KS domain that functions as a decarboxylase. This inactive KS is in most instances called KS^Q, where the superscript is the single-letter abbreviation for the amino acid (glutamine) that is present instead of the active site cysteine required for ketosynthase activity. The epothilone PKS loading domain contains a KS^Y domain not present in other PKS enzymes for which amino acid sequence is currently available in which the amino acid tyrosine has replaced the cysteine. The present invention provides recombinant DNA coding sequences for this novel KS domain.

Another important variation in PKS enzymes relates to the type of building block incorporated. Some polyketides, including epothilone, incorporate an amino acid derived building block. PKS enzymes that make such polyketides require specialized modules for incorporation. Such modules are called non-ribosomal peptide synthetase (NRPS) modules. The epothilone PKS, for example, contains an NRPS module. Another example of a variation relates to additional activities in a module. For example, one module of the

epothilone PKS contains a methyltransferase (MT) domain, a heretofore unknown domain of PKS enzymes that make modular polyketides.

The complete nucleotide sequence of the coding sequence of the open reading frames (ORFs) of the epothilone PKS genes and epothilone tailoring (modification) enzyme genes is provided in Example 1, below. This sequence information together with the information provided below regarding the locations of the open reading frames of the genes within that sequence provides the amino acid sequence of the encoded proteins. Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given amino acid sequence of the invention. The native DNA sequence encoding the epothilone PKS and epothilone modification enzymes of *Sorangium cellulosum* is shown herein merely to illustrate a preferred embodiment of the invention. The present invention includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the invention. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity and, in some instances, even an improvement of a desired activity. The present invention includes such polypeptides with alternate amino acid sequences, and the amino acid sequences shown merely illustrate preferred embodiments of the invention.

The present invention provides recombinant genes for the production of epothilones. The invention is exemplified by the cloning, characterization, and manipulation of the epothilone PKS and modification enzymes of *Sorangium cellulosum* SMP44. The description of the invention and the recombinant vectors deposited in connection with that description enable the identification, cloning, and manipulation of epothilone PKS and modification enzymes from any naturally occurring host cell that produces an epothilone. Such host cells include other *S. cellulosum* strains, such as So ce 90, other *Sorangium* species, and non-*Sorangium* cells. Such identification, cloning, and characterization can be conducted by those of ordinary skill in accordance with the present invention using standard methodology for identifying homologous DNA sequences and for identifying genes that encode a protein of function similar to a known

protein. Moreover, the present invention provides recombinant epothilone PKS and modification enzyme genes that are synthesized de novo or are assembled from non-epothilone PKS genes to provide an ordered array of domains and modules in one or more proteins that assemble to form a PKS that produces epothilone or an epothilone derivative.

The recombinant nucleic acids, proteins, and peptides of the invention are many and diverse. To facilitate an understanding of the invention and the diverse compounds and methods provided thereby, the following discussion describes various regions of the epothilone PKS and corresponding coding sequences. This discussion begins with a general discussion of the genes that encode the PKS, the location of the various domains and modules in those genes, and the location of the various domains in those modules. Then, a more detailed discussion follows, focusing first on the loading domain, followed by the NRPS module, and then the remaining eight modules of the epothilone PKS.

There are six epothilone PKS genes. The *epoA* gene encodes the 149 kDa loading domain (which can also be referred to as a loading module). The *epoB* gene encodes module 1, the 158 kDa NRPS module. The *epoC* gene encodes the 193 kDa module 2. The *epoD* gene encodes a 765 kDa protein that comprises modules 3 through 6, inclusive. The *epoE* gene encodes a 405 kDa protein that comprises modules 7 and 8. The *epoF* gene encodes a 257 kDa protein that comprises module 9 and the thioesterase domain. Immediately downstream of the *epoF* gene is *epoK*, the P450 epoxidase gene which encodes a 47 kDa protein, followed immediately by the *epoL* gene, which may encode a 24 kDa dehydratase. The *epoL* gene is followed by a number of ORFs that include genes believed to encode proteins involved in transport and regulation.

sub B2

The sequences of these genes are shown in Example 1 in one contiguous sequence or contig of 71,989 nucleotides. This contig also contains two genes that appear to originate from a transposon and are identified below as ORF A and ORF B. These two genes are believed not to be involved in epothilone biosynthesis but could possibly contain sequences that function as a promoter or enhancer. The contig also contains more than 12 additional ORFs, only 12 of which, designated ORF2 through ORF12 and ORF2 complement, are identified below. As noted, ORF2 actually is two ORFs, because the

complement of the strand shown also comprises an ORF. The function of the corresponding gene product, if any, of these ORFs has not yet been established. The Table below provides the location of various open reading frames, module-coding sequences, and domain encoding sequences within the contig sequence shown in Example 1. Those of skill in the art will recognize, upon consideration of the sequence shown in Example 1, that the actual start locations of several of the genes could differ from the start locations shown in the table, because of the presence in frame codons for methionine or valine in close proximity to the codon indicated as the start codon. The actual start codon can be confirmed by amino acid sequencing of the proteins expressed from the genes.

Start	Stop	Comment
3	992	transposase gene ORF A, not part of the PKS
989	1501	transposase gene ORF B, not part of the PKS
1998	6263	*epoA* gene, encodes the loading domain
2031	3548	KS^Y of the loading domain
3621	4661	AT of the loading domain
4917	5810	ER of the loading domain, potentially involved in formation of the thiazole moiety
5856	6155	ACP of the loading domain
6260	10493	*epoB* gene, encodes module 1, the NRPS module
6620	6649	condensation domain C2 of the NRPS module
6861	6887	heterocyclization signature sequence
6962	6982	condensation domain C4 of the NRPS module
7358	7366	condensation domain C7 (partial) of the NRPS module
7898	7921	adenylation domain A1 of the NRPS module
8261	8308	adenylation domain A3 of the NRPS module
8411	8422	adenylation domain A4 of the NRPS module
8861	8905	adenylation domain A6 of the NRPS module
8966	8983	adenylation domain A7 of the NRPS module
9090	9179	adenylation domain A8 of the NRPS module
9183	9992	oxidation region for forming thiazole
10121	10138	Adenylation domain A10 of the NRPS module

Start	Stop	Comment
10261	10306	Thiolation domain (PCP) of the NRPS module
10639	16137	*epoC* gene, encodes module 2
10654	12033	KS2, the KS domain of module 2
12250	13287	AT2, the AT domain of module 2
13327	13899	DH2, the DH domain of module 2
14962	15756	KR2, the KR domain of module 2
15763	16008	ACP2, the ACP domain of module 2
16134	37907	*epoD* gene, encodes modules 3-6
16425	17606	KS3
17817	18857	AT3
19581	20396	KR3
20424	20642	ACP3
20706	22082	KS4
22296	23336	AT4
24069	24647	KR4
24867	25151	ACP4
25203	26576	KS5
26793	27833	AT5
27966	28574	DH5
29433	30287	ER5
30321	30869	KR5
31077	31373	ACP5
31440	32807	KS6
33018	34067	AT6
34107	34676	DH6
35760	36641	ER6
36705	37256	KR6
37470	37769	ACP6
37912	49308	*epoE* gene, encodes modules 7 and 8
38014	39375	KS7
39589	40626	AT7
41341	41922	KR7
42181	42423	ACP7

Start	Stop	Comment
42478	43851	KS8
44065	45102	AT8
45262	45810	DH (inactive)
46072	47172	MT8, the methyltransferase domain of module 8
48103	48636	KR8, this domain is inactive
48850	49149	ACP8
49323	56642	*epoF* gene, encodes module 9 and the TE domain
49416	50774	KS9
50985	52025	AT9
52173	53414	DH (inactive)
54747	55313	KR9
55593	55805	ACP9
55878	56600	TE9, the thioesterase domain
56757	58016	*epoK* gene, encodes the P450 epoxidase
58194	58733	*epoL* gene (putative dehydratase)
59405	59974	ORF2 complement, complement of strand shown
59460	60249	ORF2
60271	60738	ORF3, complement of strand shown
61730	62647	ORF4 (putative transporter)
63725	64333	ORF5
64372	65643	ORF6
66237	67472	ORF7 (putative oxidoreductase)
67572	68837	ORF8 (putative oxidoreductase membrane subunit)
68837	69373	ORF9
69993	71174	ORF10 (putative transporter)
71171	71542	ORF11
71557	71989	ORF12

With this overview of the organization and sequence of the epothilone gene cluster, one can better appreciate the many different recombinant DNA compounds provided by the present invention.

The epothilone PKS is multiprotein complex composed of the gene products of the *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* genes. To confer the ability to produce epothilones to a host cell, one provides the host cell with the recombinant *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* genes of the present invention, and optionally other genes, capable of expression in that host cell. Those of skill in the art will appreciate that, while the epothilone and other PKS enzymes may be referred to as a single entity herein, these enzymes are typically multisubunit proteins. Thus, one can make a derivative PKS (a PKS that differs from a naturally occurring PKS by deletion or mutation) or hybrid PKS (a PKS that is composed of portions of two different PKS enzymes) by altering one or more genes that encode one or more of the multiple proteins that constitute the PKS.

The post-PKS modification or tailoring of epothilone includes multiple steps mediated by multiple enzymes. These enzymes are referred to herein as tailoring or modification enzymes. Surprisingly, the products of the domains of the epothilone PKS predicted to be functional by analysis of the genes that encode them are compounds that have not been previously reported. These compounds are referred to herein as epothilones G and H. Epothilones G and H lack the C-12-C-13 π-bond of epothilones C and D and the C-12-C-13 epoxide of epothilones A and B, having instead a hydrogen and hydroxyl group at C-13, a single bond between C-12 and C-13, and a hydrogen and H or methyl group at C-12. These compounds are predicted to result from the epothilone PKS, because the DNA and corresponding amino acid sequence for module 4 of the epothilone PKS does not appear to include a DH domain.

As described below, however, expression of the epothilone PKS genes *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* in certain heterologous host cells that do not express *epoK* or *epoL* leads to the production of epothilones C and D, which lack the C-13 hydroxyl and have a double bond between C-12 and C-13. The dehydration reaction that mediates the formation of this double bond may be due to the action of an as yet unrecognized domain of the epothilone PKS (for example, dehydration could occur in the next module, which possesses an active DH domain and could generate a conjugated diene precursor prior to its dehydrogenation by an ER domain) or an endogenous enzyme in the heterologous host cells (*Streptomyces coelicolor*) in which it was observed. In the

latter event, epothilones G and H may be produced in *Sorangium cellulosum* or other host cells and, to be converted to epothilones C and D, by the action of a dehydratase, which may be encoded by the *epoL* gene. In any event, epothilones C and D are converted to epothilones A and B by an epoxidase encoded by the *epoK* gene. Epothilones A and B are converted to epothilones E and F by a hydroxylase gene, which may be encoded by one of the ORFs identified above or by another gene endogenous to *Sorangium cellulosum*. Thus, one can produce an epothilone or epothilone derivative modified as desired in a host cell by providing that host cell with one or more of the recombinant modification enzyme genes provided by the invention or by utilizing a host cell that naturally expresses (or does not express) the modification enzyme. Thus, in general, by utilizing the appropriate host and by appropriate inactivation, if desired, of modification enzymes, one may interrupt the progression of G → C → A → E or the corresponding downstream processing of epothilone H at any desired point; by controlling methylation, one or both of the pathways can be selected.

Thus, the present invention provides a wide variety of recombinant DNA compounds and host cells for expressing the naturally occurring epothilones A, B, C, and D and derivatives thereof. The invention also provides recombinant host cells, particularly *Sorangium cellulosum* host cells that produce epothilone derivatives modified in a manner similar to epothilones E and F. Moreover, the invention provides host cells that can produce the heretofore unknown epothilones G and H, either by expression of the epothilone PKS genes in host cells that do not express the dehydratase that converts epothilones G and H to C and D or by mutating or altering the PKS to abolish the dehydratase function, if it is present in the epothilone PKS.

The macrolide compounds that are products of the PKS cluster can thus be modified in various ways. In addition to the modifications described above, the PKS products can be glycosylated, hydroxylated, dehydroxylated, oxidized, methylated and demethylated using appropriate enzymes. Thus, in addition to modifying the product of the PKS cluster by altering the number, functionality, or specificity of the modules contained in the PKS, additional compounds within the scope of the invention can be produced by additional enzyme-catalyzed activity either provided by a host cell in which

the polyketide synthases are produced or by modifying these cells to contain additional enzymes or by additional *in vitro* modification using purified enzymes or crude extracts or, indeed, by chemical modification.

The present invention also provides a wide variety of recombinant DNA compounds and host cells that make epothilone derivatives. As used herein, the phrase "epothilone derivative" refers to a compound that is produced by a recombinant epothilone PKS in which at least one domain has been either rendered inactive, mutated to alter its catalytic function, or replaced by a domain with a different function or in which a domain has been inserted. In any event, the "epothilone derivative PKS" functions to produce a compound that differs in structure from a naturally occurring epothilone but retains its ring backbone structure and so is called an "epothilone derivative." To faciliate a better understanding of the recombinant DNA compounds and host cells provided by the invention, a detailed discussion of the loading domain and each of the modules of the epothilone PKS, as well as novel recombinant derivatives thereof, is provided below.

sub B3

The loading domain of the epothilone PKS includes an inactive KS domain, KS^Y, an AT domain specific for malonyl CoA (which is believed to be decarboxylated by the KS^Y domain to yield an acetyl group) and an ACP domain. The present invention provides recombinant DNA compounds that encode the epothilone loading domain. The loading domain coding sequence is contained within an ~8.3 kb EcoRI restriction fragment of cosmid pKOS35-70.8A3. The KS domain is referred to as inactive, because the active site region "TAYSSSL" of the KS domain of the loading domain has a Y residue in place of the cysteine required for ketosynthase activity; this domain does have decarboxylase activity. See Witkowski *et al.*, 7 Sep. 1999, Biochem. 38(36): 11643-11650, incorporated herein by reference.

The presence of the Y residue in place of a Q residue (which occurs typically in an inactive loading domain KS) may make the KS domain less efficient at decarboxylation. The present invention provides a recombinant epothilone PKS loading domain and corresponding DNA sequences that encode an epothilone PKS loading domain in which the Y residue has been changed to a Q residue by changing the codon

therefor in the coding sequence of the loading domain. The present invention also provides recombinant PKS enzymes comprising such loading domains and host cells for producing such enzymes and the polyketides produced thereby. These recombinant loading domains include those in which just the Y residue has been changed, those in which amino acids surrounding and including the Y domain have been changed, and those in which the complete KS^Y domain has been replaced by a complete KS^Q domain. The latter embodiment includes but is not limited to a recombinant epothilone loading domain in which the KS^Y domain has been replaced by the KS^Q domain of the oleandolide PKS or the narbonolide PKS (see the references cited below in connection with the oleandomycin, narbomycin, and picromycin PKS and modification enzymes).

Sub B4

~~The epothilone loading domain also contains an AT domain believed to bind malonyl CoA. The sequence "QTAFTQPALFTFEYALAALW. . . GHSIG" in the AT domain is consistent with malonyl CoA specificity. As noted above, the malonyl CoA is believed to be decarboxylated by the KS^Y domain to yield acetyl CoA. The present invention provides recombinant epothilone derivative loading domains or their encoding DNA sequences in which the malonyl specific AT domain or its encoding sequence has been changed to another specificity, such as methylmalonyl CoA, ethylmalonyl CoA, and 2-hydroxymalonyl CoA. When expressed with the other proteins of the epothilone PKS, such loading domains lead to the production of epothilones in which the methyl substituent of the thiazole ring of epothilone is replaced with, respectively, ethyl, propyl, and hydroxymethyl. The present invention provides recombinant PKS enzymes comprising such loading domains and host cells for producing such enzymes and the polyketides produced thereby.~~

Those of skill in the art will recognize that an AT domain that is specific for 2-hydroxymalonyl CoA will result in a polyketide with a hydroxyl group at the corresponding location in the polyketide produced, and that the hydroxyl group can be methylated to yield a methoxy group by polyketide modification enzymes. See, e.g., the patent applications cited in connection with the FK-520 PKS in the table below. Consequently, reference to a PKS that has a 2-hydroxymalonyl specific AT domain

herein similarly refers to polyketides produced by that PKS that have either a hydroxyl or methoxyl group at the corresponding location in the polyketide.

The loading domain of the epothilone PKS also comprises an ER domain. While, this ER domain may be involved in forming one of the double bonds in the thiazole moiety in epothilone (in the reverse of its normal reaction), or it may be non-functional. In either event, the invention provides recombinant DNA compounds that encode the epothilone PKS loading domain with and without the ER region, as well as hybrid loading domains that contain an ER domain from another PKS (either active or inactive, with or without accompanying KR and DH domains) in place of the ER domain of the epothilone loading domain. The present invention also provides recombinant PKS enzymes comprising such loading domains and host cells for producing such enzymes and the polyketides produced thereby.

The recombinant nucleic acid compounds of the invention that encode the loading domain of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone loading domain is coexpressed with the proteins of a heterologous PKS. As used herein, reference to a heterologous modular PKS (or to the coding sequence therefor) refers to all or part of a PKS, including each of the multiple proteins constituting the PKS, that synthesizes a polyketide other than an epothilone or epothilone derivative (or to the coding sequences therefor). This coexpression can be in one of two forms. The epothilone loading domain can be coexpressed as a discrete protein with the other proteins of the heterologous PKS or as a fusion protein in which the loading domain is fused to one or more modules of the heterologous PKS. In either event, the hybrid PKS formed, in which the loading domain of the heterologous PKS is replaced by the epothilone loading domain, provides a novel PKS. Examples of a heterologous PKS that can be used to prepare such hybrid PKS enzymes of the invention include but are not limited to DEBS and the picromycin (narbonolide), oleandolide, rapamycin, FK-506, FK-520, rifamycin, and avermectin PKS enzymes and their corresponding coding sequences.

In another embodiment, a nucleic acid compound comprising a sequence that encodes the epothilone loading domain is coexpressed with the proteins that constitute the remainder of the epothilone PKS (i.e., the *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* gene products) or a recombinant epothilone PKS that produces an epothilone derivative due to an alteration or mutation in one or more of the *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* genes. As used herein, reference to an epothilone or a PKS that produces an epothilone derivative (or to the coding sequence therefor) refers to all or any one of the proteins that comprise the PKS (or to the coding sequences therefor).

In another embodiment, the invention provides recombinant nucleic acid compounds that encode a loading domain composed of part of the epothilone loading domain and part of a heterologous PKS. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT. This replacement, like the others described herein, is typically mediated by replacing the coding sequences therefor to provide a recombinant DNA compound of the invention; the recombinant DNA is used to prepare the corresponding protein. Such changes (including not only replacements but also deletions and insertions) may be referred to herein either at the DNA or protein level.

The compounds of the invention also include those in which both the KS^Y and AT domains of the epothilone loading domain have been replaced but the ACP and/or linker regions of the epothilone loading domain are left intact. Linker regions are those segments of amino acids between domains in the loading domain and modules of a PKS that help form the tertiary structure of the protein and are involved in correct alignment and positioning of the domains of a PKS. These compounds include, for example, a recombinant loading domain coding sequence in which the KS^Y and AT domain coding sequences of the epothilone PKS have been replaced by the coding sequences for the KS^Q and AT domains of, for example, the oleandolide PKS or the narbonolide PKS. There are also PKS enzymes that do not employ a KS^Q domain but instead merely utilize an AT domain that binds acetyl CoA, propionyl CoA, or butyryl CoA (the DEBS loading domain) or isobutyryl CoA (the avermectin loading domain). Thus, the compounds of the invention also include, for example, a recombinant loading domain coding sequence in

which the KS^Y and AT domain coding sequences of the epothilone PKS have been replaced by an AT domain of the DEBS or avermectin PKS. The present invention also provides recombinant DNA compounds encoding loading domains in which the ACP domain or any of the linker regions of the epothilone loading domain has been replaced by another ACP or linker region.

Any of the above loading domain coding sequences is coexpressed with the other proteins that constitute a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide to provide a PKS of the invention. If the product desired is epothilone or an epothilone derivative, then the loading domain coding sequence is typically expressed as a discrete protein, as is the loading domain in the naturally occurring epothilone PKS. If the product desired is produced by the loading domain of the invention and proteins from one or more non-epothilone PKS enzymes, then the loading domain is expressed either as a discrete protein or as a fusion protein with one or more modules of the heterologous PKS.

The present invention also provides hybrid PKS enzymes in which the epothilone loading domain has been replaced in its entirety by a loading domain from a heterologous PKS with the remainder of the PKS proteins provided by modified or unmodified epothilone PKS proteins. The present invention also provides recombinant expression vectors and host cells for producing such enzymes and the polyketides produced thereby. In one embodiment, the heterologous loading domain is expressed as a discrete protein in a host cell that expresses the *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* gene products. In another embodiment, the heterologous loading domain is expressed as a fusion protein with the *epoB* gene product in a host cell that expresses the *epoC*, *epoD*, *epoE*, and *epoF* gene products. In a related embodiment, the present invention provides recombinant epothilone PKS enzymes in which the loading domain has been deleted and replaced by an NRPS module and corresponding recombinant DNA compounds and expression vectors. In this embodiment, the recombinant PKS enzymes thus produce an epothilone derivative that comprises a dipeptide moiety, as in the compound leinamycin. The invention provides such enzymes in which the remainder of the epothilone PKS is

identical in function to the native epothilone PKS as well as those in which the remainder is a recombinant PKS that produces an epothilone derivative of the invention.

The present invention also provides reagents and methods useful in deleting the loading domain coding sequence or any portion thereof from the chromosome of a host cell, such as *Sorangium cellulosum*, or replacing those sequences or any portion thereof with sequences encoding a recombinant loading domain. Using a recombinant vector that comprises DNA complementary to the DNA including and/or flanking the loading domain coding sequence in the *Sorangium* chromosome, one can employ the vector and homologous recombination to replace the native loading domain coding sequence with a recombinant loading domain coding sequence or to delete the sequence altogether.

Moreover, while the above discussion focuses on deleting or replacing the epothilone loading domain coding sequences, those of skill in the art will recognize that the present invention provides recombinant DNA compounds, vectors, and methods useful in deleting or replacing all or any portion of an epothilone PKS gene or an epothilone modification enzyme gene. Such methods and materials are useful for a variety of purposes. One purpose is to construct a host cell that does not make a naturally occurring epothilone or epothilone derivative. For example, a host cell that has been modified to not produce a naturally occurring epothilone may be particularly preferred for making epothilone derivatives or other polyketides free of any naturally occurring epothilone. Another purpose is to replace the deleted gene with a gene that has been altered so as to provide a different product or to produce more of one product than another.

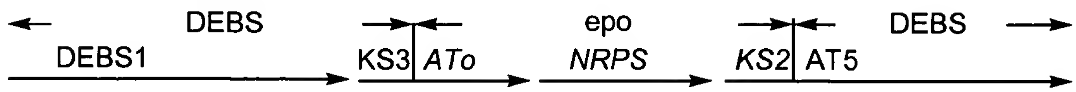
If the epothilone loading domain coding sequence has been deleted or otherwise rendered non-functional in a *Sorangium cellulosum* host cell, then the resulting host cell will produce a non-functional epothilone PKS. This PKS could still bind and process extender units, but the thiazole moiety of epothilone would not form, leading to the production of a novel epothilone derivative. Because this derivative would predictably contain a free amino group, it would be produced at most in low quantities. As noted above, however, provision of a heterologous or other recombinant loading domain to the

host cell would result in the production of an epothilone derivative with a structure determined by the loading domain provided.

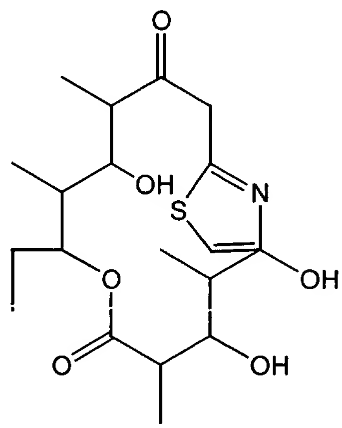
The loading domain of the epothilone PKS is followed by the first module of the PKS, which is an NRPS module specific for cysteine. This NRPS module is naturally expressed as a discrete protein, the product of the *epoB* gene. The present invention provides the *epoB* gene in recombinant form. The recombinant nucleic acid compounds of the invention that encode the NRPS module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a nucleic acid compound comprising a sequence that encodes the epothilone NRPS module is coexpressed with genes encoding one or more proteins of a heterologous PKS. The NRPS module can be expressed as a discrete protein or as a fusion protein with one of the proteins of the heterologous PKS. The resulting PKS, in which at least a module of the heterologous PKS is replaced by the epothilone NRPS module or the NRPS module is in effect added as a module to the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the epothilone NRPS module is coexpressed with the other epothilone PKS proteins or modified versions thereof to provide a recombinant epothilone PKS that produces an epothilone or an epothilone derivative.

Two hybrid PKS enzymes provided by the invention illustrate this aspect. Both hybrid PKS enzymes are hybrids of DEBS and the epothilone NRPS module. The first hybrid PKS is composed of four proteins: (i) DEBS1; (ii) a fusion protein composed of the KS domain of module 3 of DEBS and all but the KS domain of the loading domain of the epothilone PKS; (iii) the epothilone NRPS module; and (iv) a fusion protein composed of the KS domain of module 2 of the epothilone PKS fused to the AT domain of module 5 of DEBS and the rest of DEBS3. This hybrid PKS produces a novel polyketide with a thiazole moiety incorporated into the macrolactone ring and a molecular weight of 413.53 when expressed in *Streptomyces coelicolor*. Glycosylated, hydroxylated, and methylated derivatives can be produced by expression of the hybrid PKS in *Saccharopolyspora erythraea*.

Diagrammatically, the construct is represented:



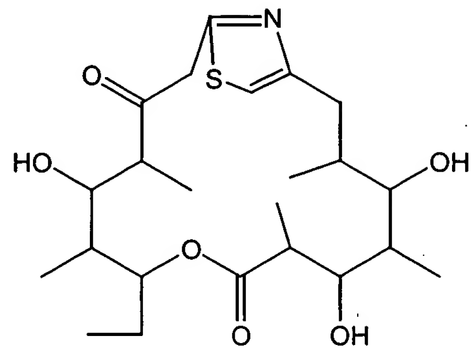
The structure of the product is:



The second hybrid PKS illustrating this aspect of the invention is composed of five proteins: (i) DEBS1; (ii) a fusion protein composed of the KS domain of module 3 of DEBS and all but the KS domain of the loading domain of the epothilone PKS; (iii) the epothilone NRPS module; and (iv) a fusion protein composed of the KS domain of module 2 of the epothilone PKS fused to the AT domain of module 4 of DEBS and the rest of DEBS2; and (v) DEBS3. This hybrid PKS produces a novel polyketide with a thiazole moiety incorporated into the macrolactone ring and a molecular weight of 455.61 when expressed in *Streptomyces coelicolor*. Glycosylated, hydroxylated, and methylated derivatives can be produced by expression of the hybrid PKS in *Saccharopolyspora erythraea*.

Diagrammatically, the construct is represented:

The structure of the product is:



In another embodiment, a portion of the NRPS module coding sequence is utilized in conjunction with a heterologous coding sequence. In this embodiment, the invention provides, for example, changing the specificity of the NRPS module of the epothilone PKS from a cysteine to another amino acid. This change is accomplished by constructing a coding sequence in which all or a portion of the epothilone PKS NRPS module coding sequences have been replaced by those coding for an NRPS module of a different specificity. In one illustrative embodiment, the specificity of the epothilone NRPS module is changed from cysteine to serine or threonine. When the thus modified NRPS module is expressed with the other proteins of the epothilone PKS, the recombinant PKS produces an epothilone derivative in which the thiazole moiety of epothilone (or an epothilone derivative) is changed to an oxazole or 5-methyloxazole moiety, respectively. Alternatively, the present invention provides recombinant PKS enzymes composed of the products of the *epoA*, *epoC*, *epoD*, *epoE*, and *epoF* genes (or modified versions thereof) without an NRPS module or with an NRPS module from a heterologous PKS. The heterologous NRPS module can be expressed as a discrete protein or as a fusion protein with either the *epoA* or *epoC* genes.

The invention also provides methods and reagents useful in changing the specificity of a heterologous NRPS module from another amino acid to cysteine. This change is accomplished by constructing a coding sequence in which the sequences that determine the specificity of the heterologous NRPS module have been replaced by those that specify cysteine from the epothilone NRPS module coding sequence. The resulting heterologous NRPS module is typically coexpressed in conjunction with the proteins

constituting a heterologous PKS that synthesizes a polyketide other than epothilone or an epothilone derivative, although the heterologous NRPS module can also be used to produce epothilone or an epothilone derivative.

In another embodiment, the invention provides recombinant epothilone PKS enzymes and corresponding recombinant nucleic acid compounds and vectors in which the NRPS module has been inactivated or deleted. Such enzymes, compounds, and vectors are constructed generally in accordance with the teaching for deleting or inactivating the epothilone PKS or modification enzyme genes above. Inactive NRPS module proteins and the coding sequences therefore provided by the invention include those in which the peptidyl carrier protein (PCP) domain has been wholly or partially deleted or otherwise rendered inactive by changing the active site serine (the site for phosphopantetheinylation) to another amino acid, such as alanine, or the adenylation domains have been deleted or otherwise rendered inactive. In one embodiment, both the loading domain and the NRPS have been deleted or rendered inactive. In any event, the resulting epothilone PKS can then function only if provided a substrate that binds to the KS domain of module 2 (or a subsequent module) of the epothilone PKS or a PKS for an epothilone derivative. In a method provided by the invention, the thus modified cells are then fed activated acylthioesters that are bound by preferably the second, but potentially any subsequent, module and processed into novel epothilone derivatives.

Thus, in one embodiment, the invention provides *Sorangium* and non-*Sorangium* host cells that express an epothilone PKS (or a PKS that produces an epothilone derivative) with an inactive NRPS. The host cell is fed activated acylthioesters to produce novel epothilone derivatives of the invention. The host cells expressing, or cell free extracts containing, the PKS can be fed or supplied with N-acylcysteamine thioesters (NACS) of novel precursor molecules to prepare epothilone derivatives. See U.S. provisional patent application Serial No. 60/117,384, filed 27 Jan. 1999, and PCT patent publication No. US99/03986, both of which are incorporated herein by reference, and Example 6, below.

KK 7/2/02

The second (first non-NRPS) module of the epothilone PKS includes a KS, an AT specific for methylmalonyl CoA, a DH, a KR, and an ACP. This module is encoded by a

sequence within an ~13.1 kb EcoRI-NsiI restriction fragment of cosmid pKOS35-70.8A3.

The recombinant nucleic acid compounds of the invention that encode the second module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. The second module of the epothilone PKS is produced as a discrete protein by the *epoC* gene. The present invention provides the *epoC* gene in recombinant form. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone second module is coexpressed with the proteins constituting a heterologous PKS either as a discrete protein or as a fusion protein with one or more modules of the heterologous PKS. The resulting PKS, in which a module of the heterologous PKS is either replaced by the second module of the epothilone PKS or the latter is merely added to the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the second module of the epothilone PKS is coexpressed with the other proteins constituting the epothilone PKS or a recombinant epothilone PKS that produces an epothilone derivative.

In another embodiment, all or only a portion of the second module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting either the DH or KR or both; replacing the DH or KR or both with a DH or KR or both that specify a different stereochemistry; and/or inserting an ER. Generally, any reference herein to inserting or replacing a PKS KR, DH, and/or ER domain includes the replacement of the associated KR, DH, or ER domains in that module, typically with corresponding domains from the module from which the inserted or replacing domain is obtained. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a gene for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous second module coding sequence can be coexpressed with the other proteins

that constitute a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. Alternatively, one can delete or replace the second module of the epothilone PKS with a module from a heterologous PKS, which can be expressed as a discrete protein or as a fusion protein fused to either the *epoB* or *epoD* gene product.

Illustrative recombinant PKS genes of the invention include those in which the AT domain encoding sequences for the second module of the epothilone PKS have been altered or replaced to change the AT domain encoded thereby from a methylmalonyl specific AT to a malonyl specific AT. Such malonyl specific AT domain encoding nucleic acids can be isolated, for example and without limitation, from the PKS genes encoding the narbonolide PKS, the rapamycin PKS (i.e., modules 2 and 12), and the FK-520 PKS (i.e., modules 3, 7, and 8). When such a hybrid second module is coexpressed with the other proteins constituting the epothilone PKS, the resulting epothilone derivative produced is a 16-desmethyl epothilone derivative.

In addition, the invention provides DNA compounds and vectors encoding recombinant epothilone PKS enzymes and the corresponding recombinant proteins in which the KS domain of the second (or subsequent) module has been inactivated or deleted. In a preferred embodiment, this inactivation is accomplished by changing the codon for the active site cysteine to an alanine codon. As with the corresponding variants described above for the NRPS module, the resulting recombinant epothilone PKS enzymes are unable to produce an epothilone or epothilone derivative unless supplied a precursor that can be bound and extended by the remaining domains and modules of the recombinant PKS enzyme. Illustrative diketides are described in Example 6, below.

The third module of the epothilone PKS includes a KS, an AT specific for malonyl CoA, a KR, and an ACP. This module is encoded by a sequence within an ~8 kb BglII-NsiI restriction fragment of cosmid pKOS35-70.8A3.

The recombinant DNA compounds of the invention that encode the third module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. The third module of the epothilone PKS is expressed in a protein, the product of the *epoD* gene, which also contains modules 4, 5, and 6. The present invention provides the *epoD* gene in recombinant form. The present invention

also provides recombinant DNA compounds that encode each of the epothilone PKS modules 3, 4, 5, and 6, as discrete coding sequences without coding sequences for the other epothilone modules. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone third module is coexpressed with proteins constituting a heterologous PKS. The third module of the epothilone PKS can be expressed either as a discrete protein or as a fusion protein fused to one or more modules of the heterologous PKS. The resulting PKS, in which a module of the heterologous PKS is either replaced by that for the third module of the epothilone PKS or the latter is merely added to the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the third module of the epothilone PKS is coexpressed with proteins comprising the remainder of the epothilone PKS or a recombinant epothilone PKS that produces an epothilone derivative, typically as a protein comprising not only the third but also the fourth, fifth, and sixth modules.

In another embodiment, all or a portion of the third module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR; replacing the KR with a KR that specifies a different stereochemistry; and/or inserting a DH or a DH and an ER. As above, the reference to inserting a DH or a DH and an ER includes the replacement of the KR with a DH and KR or an ER, DH, and KR. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous third module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide.

Illustrative recombinant PKS genes of the invention include those in which the AT domain encoding sequences for the third module of the epothilone PKS have been altered or replaced to change the AT domain encoded thereby from a malonyl specific

AT to a methylmalonyl specific AT. Such methylmalonyl specific AT domain encoding nucleic acids can be isolated, for example and without limitation, from the PKS genes encoding DEBS, the narbonolide PKS, the rapamycin PKS, and the FK-520 PKS. When coexpressed with the remaining modules and proteins of the epothilone PKS or an epothilone PKS derivative, the recombinant PKS produces the 14-methyl epothilone derivatives of the invention.

Those of skill in the art will recognize that the KR domain of the third module of the PKS is responsible for forming the hydroxyl group involved in cyclization of epothilone. Consequently, abolishing the KR domain of the third module or adding a DH or DH and ER domains will interfere with the cyclization, leading either to a linear molecule or to a molecule cyclized at a different location than is epothilone.

The fourth module of the epothilone PKS includes a KS, an AT that can bind either malonyl CoA or methylmalonyl CoA, a KR, and an ACP. This module is encoded by a sequence within an ~10 kb NsiI-HindIII restriction fragment of cosmid pKOS35-70.1A2.

The recombinant DNA compounds of the invention that encode the fourth module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone fourth module is inserted into a DNA compound that comprises the coding sequence for one or more modules of a heterologous PKS. The resulting construct encodes a protein in which a module of the heterologous PKS is either replaced by that for the fourth module of the epothilone PKS or the latter is merely added to the modules of the heterologous PKS. Together with other proteins that constitute the heterologous PKS, this protein provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the fourth module of the epothilone PKS is expressed in a host cell that also expresses the remaining modules and proteins of the epothilone PKS or a recombinant epothilone PKS that produces an epothilone derivative. For making epothilone or epothilone derivatives, the recombinant fourth module is usually expressed in a protein that also contains the epothilone third, fifth, and sixth modules or modified versions thereof.

In another embodiment, all or a portion of the fourth module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA and methylmalonyl specific AT with a malonyl CoA, methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR; and/or replacing the KR, including, optionally, to specify a different stereochemistry; and/or inserting a DH or a DH and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a gene for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous fourth module coding sequence is incorporated into a protein subunit of a recombinant PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. If the desired polyketide is an epothilone or epothilone derivative, the recombinant fourth module is typically expressed as a protein that also contains the third, fifth, and sixth modules of the epothilone PKS or modified versions thereof. Alternatively, the invention provides recombinant PKS enzymes for epothilones and epothilone derivatives in which the entire fourth module has been deleted or replaced by a module from a heterologous PKS.

In a preferred embodiment, the invention provides recombinant DNA compounds comprising the coding sequence for the fourth module of the epothilone PKS modified to encode an AT that binds methylmalonyl CoA and not malonyl CoA. These recombinant molecules are used to express a protein that is a recombinant derivative of the *epoD* protein that comprises the modified fourth module as well as modules 3, 5, and 6, any one or more of which can optionally be in derivative form, of the epothilone PKS. In another preferred embodiment, the invention provides recombinant DNA compounds comprising the coding sequence for the fourth module of the epothilone PKS modified to encode an AT that binds malonyl CoA and not methylmalonyl CoA. These recombinant molecules are used to express a protein that is a recombinant derivative of the *epoD* protein that comprises the modified fourth module as well as modules 3, 5, and 6, any one or more of which can optionally be in derivative form, of the epothilone PKS.

Prior to the present invention, it was known that *Sorangium cellulosum* produced epothilones A, B, C, D, E, and F and that epothilones A, C, and E had a hydrogen at C-12, while epothilones B, D, and F had a methyl group at this position. Unappreciated prior to the present invention was the order in which these compounds were synthesized in *S. cellulosum*, and the mechanism by which some of the compounds had a hydrogen at C-12 where others had a methyl group at this position. The present disclosure reveals that epothilones A and B are derived from epothilones C and D by action of the *epoK* gene product and that the presence of a hydrogen or methyl moiety at C-12 is due to the AT domain of module 4 of the epothilone PKS. This domain can bind either malonyl or methylmalonyl CoA and, consistent with its having greater similarity to malonyl specific AT domains than to methylmalonyl specific AT domains, binds malonyl CoA more often than methylmalonyl CoA.

Thus, the invention provides recombinant DNA compounds and expression vectors and the corresponding recombinant PKS in which the hybrid fourth module with a methylmalonyl specific AT has been incorporated. The methylmalonyl specific AT coding sequence can originate, for example and without limitation, from coding sequences for the oleandolide PKS, DEBS, the narbonolide PKS, the rapamycin PKS, or any other PKS that comprises a methylmalonyl specific AT domain. In accordance with the invention, the hybrid fourth module expressed from this coding sequence is incorporated into the epothilone PKS (or the PKS for an epothilone derivative), typically as a derivative *epoD* gene product. The resulting recombinant epothilone PKS produces epothilones with a methyl moiety at C-12, i.e., epothilone H (or an epothilone H derivative) if there is no dehydratase activity to form the C-12-C-13 alkene; epothilone D (or an epothilone D derivative), if the dehydratase activity but not the epoxidase activity is present; epothilone B (or an epothilone B derivative), if both the dehydratase and epoxidase activity but not the hydroxylase activity are present; and epothilone F (or an epothilone F derivative), if all three dehydratase, epoxidase, and hydroxylase activities are present. As indicated parenthetically above, the cell will produce the corresponding epothilone derivative if there have been other changes to the epothilone PKS.

If the recombinant PKS comprising the hybrid methylmalonyl specific fourth module is expressed in, for example, *Sorangium cellulosum*, the appropriate modifying enzymes are present (unless they have been rendered inactive in accordance with the methods herein), and epothilones D, B, and/or F are produced. Such production is typically carried out in a recombinant *S. cellulosum* provided by the present invention in which the native epothilone PKS is unable to function at all or unable to function except in conjunction with the recombinant fourth module provided. In an illustrative example, one can use the methods and reagents of the invention to render inactive the *epoD* gene in the native host. Then, one can transform that host with a vector comprising the recombinant *epoD* gene containing the hybrid fourth module coding sequence. The recombinant vector can exist as an extrachromosomal element or as a segment of DNA integrated into the host cell chromosome. In the latter embodiment, the invention provides that one can simply integrate the recombinant methylmalonyl specific module 4 coding sequence into wild-type *S. cellulosum* by homologous recombination with the native *epoD* gene to ensure that only the desired epothilone is produced. The invention provides that the *S. cellulosum* host can either express or not express (by mutation or homologous recombination of the native genes therefor) the dehydratase, epoxidase, and/or oxidase gene products and thus form or not form the corresponding epothilone D, B, and F compounds, as the practitioner elects.

Sorangium cellulosum modified as described above is only one of the recombinant host cells provided by the invention. In a preferred embodiment, the recombinant methylmalonyl specific epothilone fourth module coding sequences are used in accordance with the methods of invention to produce epothilone D, B, and F (or their corresponding derivatives) in heterologous host cells. Thus, the invention provides reagents and methods for introducing the epothilone or epothilone derivative PKS and epothilone dehydratase, epoxidase, and hydroxylase genes and combinations thereof into heterologous host cells.

The recombinant methylmalonyl specific epothilone fourth module coding sequences provided by the invention afford important alternative methods for producing desired epothilone compounds in host cells. Thus, the invention provides a hybrid fourth

module coding sequence in which, in addition to the replacement of the endogenous AT coding sequence with a coding sequence for an AT specific for methylmalonyl Co A, coding sequences for a DH and KR for, for example and without limitation, module 10 of the rapamycin PKS or modules 1 or 5 of the FK-520 PKS have replaced the endogenous KR coding sequences. When the gene product comprising the hybrid fourth module and epothilone PKS modules 3, 5, and 6 (or derivatives thereof) encoded by this coding sequence is incorporated into a PKS comprising the other epothilone PKS proteins (or derivatives thereof) produced in a host cell, the cell makes either epothilone D or its trans stereoisomer (or derivatives thereof), depending on the stereochemical specificity of the inserted DH and KR domains.

Similarly, and as noted above, the invention provides recombinant DNA compounds comprising the coding sequence for the fourth module of the epothilone PKS modified to encode an AT that binds malonyl CoA and not methylmalonyl CoA. The invention provides recombinant DNA compounds and vectors and the corresponding recombinant PKS in which this hybrid fourth module has been incorporated into a derivative *epoD* gene product. When incorporated into the epothilone PKS (or the PKS for an epothilone derivative), the resulting recombinant epothilone PKS produces epothilones C, A, and E, depending, again, on whether epothilone modification enzymes are present. As noted above, depending on the host, whether the fourth module includes a KR and DH domain, and on whether and which of the dehydratase, epoxidase, and oxidase activities are present, the practitioner of the invention can produce one or more of the epothilone G, C, A, and E compounds and derivatives thereof using the compounds, host cells, and methods of the invention.

The fifth module of the epothilone PKS includes a KS, an AT that binds malonyl CoA, a DH, an ER, a KR, and an ACP. This module is encoded by a sequence within an ~12.4 kb NsiI-NotI restriction fragment of cosmid pKOS35-70.1A2.

The recombinant DNA compounds of the invention that encode the fifth module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone fifth module is inserted into a DNA compound that comprises

the coding sequence for one or more modules of a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the fifth module of the epothilone PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, can be incorporated into an expression vector and used to produce the recombinant protein encoded thereby. When the recombinant protein is combined with the other proteins of the heterologous PKS, a novel PKS is produced. In another embodiment, a DNA compound comprising a sequence that encodes the fifth module of the epothilone PKS is inserted into a DNA compound that comprises coding sequences for the epothilone PKS or a recombinant epothilone PKS that produces an epothilone derivative. In the latter constructs, the epothilone fifth module is typically expressed as a protein comprising the third, fourth, and sixth modules of the epothilone PKS or derivatives thereof.

In another embodiment, a portion of the fifth module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module coding sequence and the hybrid module encoded thereby. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one, two, or all three of the ER, DH, and KR; and/or replacing any one, two, or all three of the ER, DH, and KR with either a KR, a DH and KR, or a KR, DH, and ER, including, optionally, to specify a different stereochemistry. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting hybrid fifth module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. Alternatively, the fifth module of the epothilone PKS can be deleted or replaced in its entirety by a module of a heterologous PKS to produce a protein that in combination with the other proteins of the epothilone PKS or derivatives thereof constitutes a PKS that produces an epothilone derivative.

Illustrative recombinant PKS genes of the invention include recombinant *epoD* gene derivatives in which the AT domain encoding sequences for the fifth module of the epothilone PKS have been altered or replaced to change the AT domain encoded thereby from a malonyl specific AT to a methylmalonyl specific AT. Such methylmalonyl specific AT domain encoding nucleic acids can be isolated, for example and without limitation, from the PKS genes encoding DEBS, the narbonolide PKS, the rapamycin PKS, and the FK-520 PKS. When such recombinant *epoD* gene derivatives are coexpressed with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes (or derivatives thereof), the PKS composed thereof produces the 10-methyl epothilones or derivatives thereof. Another recombinant *epoD* gene derivative provided by the invention includes not only this altered module 5 coding sequence but also module 4 coding sequences that encode an AT domain that binds only methylmalonyl CoA. When incorporated into a PKS with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes, the recombinant *epoD* gene derivative product leads to the production of 10-methyl epothilone B and/or D derivatives.

Other illustrative recombinant *epoD* gene derivatives of the invention include those in which the ER, DH, and KR domain encoding sequences for the fifth module of the epothilone PKS have been replaced with those encoding (i) a KR and DH domain; (ii) a KR domain; and (iii) an inactive KR domain. These recombinant *epoD* gene derivatives of the invention are coexpressed with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes to produce a recombinant PKS that makes the corresponding (i) C-11 alkene, (ii) C-11 hydroxy, and (iii) C-11 keto epothilone derivatives. These recombinant *epoD* gene derivatives can also be coexpressed with recombinant epo genes containing other alterations or can themselves be further altered to produce a PKS that makes the corresponding C-11 epothilone derivatives. For example, one recombinant *epoD* gene derivative provided by the invention also includes module 4 coding sequences that encode an AT domain that binds only methylmalonyl CoA. When incorporated into a PKS with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes, the recombinant *epoD* gene derivative product leads to the production of the corresponding C-11 epothilone B and/or D derivatives.

Functionally similar *epoD* genes for producing the epothilone C-11 derivatives can also be made by inactivation of one, two, or all three of the ER, DH, and KR domains of the epothilone fifth module. However, the preferred mode for altering such domains in any module is by replacement with the complete set of desired domains taken from another module of the same or a heterologous PKS coding sequence. In this manner, the natural architecture of the PKS is conserved. Also, when present, KR and DH or KR, DH, and ER domains that function together in a native PKS are preferably used in the recombinant PKS. Illustrative replacement domains for the substitutions described above include, for example and without limitation, the inactive KR domain from the rapamycin PKS module 3 to form the ketone, the KR domain from the rapamycin PKS module 5 to form the alcohol, and the KR and DH domains from the rapamycin PKS module 4 to form the alkene. Other such inactive KR, active KR, and active KR and DH domain encoding nucleic acids can be isolated from, for example and without limitation, the PKS genes encoding DEBS, the narbonolide PKS, and the FK-520 PKS. Each of the resulting PKS enzymes produces a polyketide compound that comprises a functional group at the C-11 position that can be further derivatized *in vitro* by standard chemical methodology to yield semi-synthetic epothilone derivatives of the invention.

The sixth module of the epothilone PKS includes a KS, an AT that binds methylmalonyl CoA, a DH, an ER, a KR, and an ACP. This module is encoded by a sequence within an ~14.5 kb HindIII-NsiI restriction fragment of cosmid pKOS35-70.1A2.

The recombinant DNA compounds of the invention that encode the sixth module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone sixth module is inserted into a DNA compound that comprises the coding sequence for one or more modules of a heterologous PKS. The resulting protein encoded by the construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the sixth module of the epothilone PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS when coexpressed with the other proteins

comprising the PKS. In another embodiment, a DNA compound comprising a sequence that encodes the sixth module of the epothilone PKS is inserted into a DNA compound that comprises the coding sequence for modules 3, 4, and 5 of the epothilone PKS or a recombinant epothilone PKS that produces an epothilone derivative and coexpressed with the other proteins of the epothilone or epothilone derivative PKS to produce a PKS that makes epothilone or an epothilone derivative in a host cell.

In another embodiment, a portion of the sixth module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one, two, or all three of the ER, DH, and KR; and/or replacing any one, two, or all three of the ER, DH, and KR with either a KR, a DH and KR, or a KR, DH, and ER, including, optionally, to specify a different stereochemistry. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous sixth module coding sequence can be utilized in conjunction with a coding sequence for a protein subunit of a PKS that makes epothilone, an epothilone derivative, or another polyketide. If the PKS makes epothilone or an epothilone derivative, the hybrid sixth module is typically expressed as a protein comprising modules 3, 4, and 5 of the epothilone PKS or derivatives thereof. Alternatively, the sixth module of the epothilone PKS can be deleted or replaced in its entirety by a module from a heterologous PKS to produce a PKS for an epothilone derivative.

Illustrative recombinant PKS genes of the invention include those in which the AT domain encoding sequences for the sixth module of the epothilone PKS have been altered or replaced to change the AT domain encoded thereby from a methylmalonyl specific AT to a malonyl specific AT. Such malonyl specific AT domain encoding nucleic acids can be isolated from, for example and without limitation, the PKS genes

encoding the narbonolide PKS, the rapamycin PKS, and the FK-520 PKS. When a recombinant *epoD* gene of the invention encoding such a hybrid module 6 is coexpressed with the other epothilone PKS genes, the recombinant PKS makes the 8-desmethyl epothilone derivatives. This recombinant *epoD* gene derivative can also be coexpressed with recombinant epo gene derivatives containing other alterations or can itself be further altered to produce a PKS that makes the corresponding 8-desmethyl epothilone derivatives. For example, one recombinant *epoD* gene provided by the invention also includes module 4 coding sequences that encode an AT domain that binds only methylmalonyl CoA. When incorporated into a PKS with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes, the recombinant *epoD* gene product leads to the production of the 8-desmethyl derivatives of epothilones B and D.

Other illustrative recombinant *epoD* gene derivatives of the invention include those in which the ER, DH, and KR domain encoding sequences for the sixth module of the epothilone PKS have been replaced with those that encode (i) a KR and DH domain; (ii) a KR domain; and (iii) an inactive KR domain. These recombinant *epoD* gene derivatives of the invention, when coexpressed with the other epothilone PKS genes make the corresponding (i) C-9 alkene, (ii) C-9 hydroxy, and (iii) C-9 keto epothilone derivatives. These recombinant *epoD* gene derivatives can also be coexpressed with other recombinant epo gene derivatives containing other alterations or can themselves be further altered to produce a PKS that makes the corresponding C-9 epothilone derivatives. For example, one recombinant *epoD* gene derivative provided by the invention also includes module 4 coding sequences that encode an AT domain that binds only methylmalonyl CoA. When incorporated into a PKS with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes, the recombinant *epoD* gene product leads to the production of the C-9 derivatives of epothilones B and D.

Functionally equivalent sixth modules can also be made by inactivation of one, two, or all three of the ER, DH, and KR domains of the epothilone sixth module. The preferred mode for altering such domains in any module is by replacement with the complete set of desired domains taken from another module of the same or a heterologous PKS coding sequence. Illustrative replacement domains for the substitutions

described above include but are not limited to the inactive KR domain from the rapamycin PKS module 3 to form the ketone, the KR domain from the rapamycin PKS module 5 to form the alcohol, and the KR and DH domains from the rapamycin PKS module 4 to form the alkene. Other such inactive KR, active KR, and active KR and DH domain encoding nucleic acids can be isolated from for example and without limitation the PKS genes encoding DEBS, the narbonolide PKS, and the FK-520 PKS. Each of the resulting PKSs produces a polyketide compound that comprises a functional group at the C-9 position that can be further derivatized *in vitro* by standard chemical methodology to yield semi-synthetic epothilone derivatives of the invention.

The seventh module of the epothilone PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, and an ACP. This module is encoded by a sequence within an ~8.7 kb BglII restriction fragment from cosmid pKOS35-70.4.

The recombinant DNA compounds of the invention that encode the seventh module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. The seventh module of the epothilone PKS is contained in the gene product of the *epoE* gene, which also contains the eighth module. The present invention provides the *epoE* gene in recombinant form, but also provides DNA compounds that encode the seventh module without coding sequences for the eighth module as well as DNA compounds that encode the eighth module without coding sequences for the seventh module. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone seventh module is inserted into a DNA compound that comprises the coding sequence for one or more modules of a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the seventh module of the epothilone PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence that can be expressed in a host cell. Alternatively, the epothilone seventh module can be expressed as a discrete protein. In another embodiment, a DNA compound comprising a sequence that encodes the seventh module of the epothilone PKS is expressed to form a protein that, together with other proteins, constitutes the epothilone PKS or a PKS that produces an epothilone derivative. In these

embodiments, the seventh module is typically expressed as a protein comprising the eighth module of the epothilone PKS or a derivative thereof and coexpressed with the *epoA*, *epoB*, *epoC*, *epoD*, and *epoF* genes or derivatives thereof to constitute the PKS.

In another embodiment, a portion or all of the seventh module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR; replacing the KR with a KR that specifies a different stereochemistry; and/or inserting a DH or a DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous seventh module coding sequence is utilized, optionally in conjunction with other coding sequences, to express a protein that together with other proteins constitutes a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. When used to prepare epothilone or an epothilone derivative, the seventh module is typically expressed as a protein comprising the eighth module or derivative thereof and coexpressed with the *epoA*, *epoB*, *epoC*, *epoD*, and *epoF* genes or derivatives thereof to constitute the PKS. Alternatively, the coding sequences for the seventh module in the *epoE* gene can be deleted or replaced by those for a heterologous module to prepare a recombinant *epoE* gene derivative that, together with the *epoA*, *epoB*, *epoC*, *epoD*, and *epoF* genes, can be expressed to make a PKS for an epothilone derivative.

Illustrative recombinant *epoE* gene derivatives of the invention include those in which the AT domain encoding sequences for the seventh module of the epothilone PKS have been altered or replaced to change the AT domain encoded thereby from a methylmalonyl specific AT to a malonyl specific AT. Such malonyl specific AT domain encoding nucleic acids can be isolated from for example and without limitation the PKS genes encoding the narbonolide PKS, the rapamycin PKS, and the FK-520 PKS. When

coexpressed with the other epothilone PKS genes, *epoA*, *epoB*, *epoC*, *epoD*, and *epoF*, or derivatives thereof, a PKS for an epothilone derivative with a C-6 hydrogen, instead of a C-6 methyl, is produced. Thus, if the genes contain no other alterations, the compounds produced are the 6-desmethyl epothilones.

The eighth module of the epothilone PKS includes a KS, an AT specific for methylmalonyl CoA, inactive KR and DH domains, a methyltransferase (MT) domain, and an ACP. This module is encoded by a sequence within an ~10 kb NotI restriction fragment of cosmid pKOS35-79.85.

The recombinant DNA compounds of the invention that encode the eighth module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone eighth module is inserted into a DNA compound that comprises the coding sequence for one or more modules of a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the eighth module of the epothilone PKS or the latter is merely added to coding sequences for modules of the heterologous PKS, provides a novel PKS coding sequence that is expressed with the other proteins constituting the PKS to provide a novel PKS. Alternatively, the eighth module can be expressed as a discrete protein that can associate with other PKS proteins to constitute a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the eighth module of the epothilone PKS is coexpressed with the other proteins constituting the epothilone PKS or a PKS that produces an epothilone derivative. In these embodiments, the eighth module is typically expressed as a protein that also comprises the seventh module or a derivative thereof.

In another embodiment, a portion or all of the eighth module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the inactive KR and/or the inactive DH; replacing the inactive KR and/or DH with an active KR and/or DH; and/or inserting an ER. In addition, the KS

and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous eighth module coding sequence is expressed as a protein that is utilized in conjunction with the other proteins that constitute a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. When used to prepare epothilone or an epothilone derivative, the heterologous or hybrid eighth module is typically expressed as a recombinant *epoE* gene product that also contains the seventh module. Alternatively, the coding sequences for the eighth module in the *epoE* gene can be deleted or replaced by those for a heterologous module to prepare a recombinant *epoE* gene that, together with the *epoA*, *epoB*, *epoC*, *epoD*, and *epoF* genes, can be expressed to make a PKS for an epothilone derivative.

The eighth module of the epothilone PKS also comprises a methylation or methyltransferase (MT) domain with an activity that methylates the epothilone precursor. This function can be deleted to produce a recombinant *epoD* gene derivative of the invention, which can be expressed with the other epothilone PKS genes or derivatives thereof that makes an epothilone derivative that lacks one or both methyl groups, depending on whether the AT domain of the eighth module has been changed to a malonyl specific AT domain, at the corresponding C-4 position of the epothilone molecule. In another important embodiment, the present invention provides recombinant DNA compounds that encode a polypeptide with this methylation domain and activity and a variety of recombinant PKS coding sequences that encode recombinant PKS enzymes that incorporate this polypeptide. The availability of this MT domain and the coding sequences therefor provides a significant number of new polyketides that differ from known polyketides by the presence of at least an additional methyl group. The MT domain of the invention can in effect be added to any PKS module to direct the methylation at the corresponding location in the polyketide produced by the PKS. As but one illustrative example, the present invention provides the recombinant nucleic acid compounds resulting from inserting the coding sequence for this MT activity into a

coding sequence for any one or more of the six modules of the DEBS enzyme to produce a recombinant DEBS that synthesizes a 6-deoxyerythronolide B derivative that comprises one or more additional methyl groups at the C-2, C-4, C-6, C-8, C-10, and/or C-12 positions. In such constructs, the MT domain can be inserted adjacent to the AT or the ACP.

The ninth module of the epothilone PKS includes a KS, an AT specific for malonyl CoA, a KR, an inactive DH, and an ACP. This module is encoded by a sequence within an ~14.7 HindIII-BglII kb restriction fragment of cosmid pKOS35-79.85.

The recombinant DNA compounds of the invention that encode the ninth module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. The ninth module of the epothilone PKS is expressed as a protein, the product of the *epoF* gene, that also contains the TE domain of the epothilone PKS. The present invention provides the *epoF* gene in recombinant form, as well as DNA compounds that encode the ninth module without the coding sequences for the TE domain and DNA compounds that encode the TE domain without the coding sequences for the ninth module. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone ninth module is inserted into a DNA compound that comprises the coding sequence for one or more modules of a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the ninth module of the epothilone PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS protein coding sequence that when coexpressed with the other proteins constituting a PKS provides a novel PKS. The ninth module coding sequence can also be expressed as a discrete protein with or without an attached TE domain. In another embodiment, a DNA compound comprising a sequence that encodes the ninth module of the epothilone PKS is expressed as a protein together with other proteins to constitute an epothilone PKS or a PKS that produces an epothilone derivative. In these embodiments, the ninth module is typically expressed as a protein that also contains the TE domain of either the epothilone PKS or a heterologous PKS.

In another embodiment, a portion or all of the ninth module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxy malonyl CoA specific AT; deleting the KR; replacing the KR with a KR that specifies a different stereochemistry; and/or inserting a DH or a DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous ninth module coding sequence is coexpressed with the other proteins constituting a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. Alternatively, the present invention provides a PKS for an epothilone or epothilone derivative in which the ninth module has been replaced by a module from a heterologous PKS or has been deleted in its entirety. In the latter embodiment, the TE domain is expressed as a discrete protein or fused to the eighth module.

The ninth module of the epothilone PKS is followed by a thioesterase domain. This domain is encoded in the ~14.7 kb HindIII-BglII restriction comprising the ninth module coding sequence. The present invention provides recombinant DNA compounds that encode hybrid PKS enzymes in which the ninth module of the epothilone PKS is fused to a heterologous thioesterase or one or more modules of a heterologous PKS are fused to the epothilone PKS thioesterase. Thus, for example, a thioesterase domain coding sequence from another PKS can be inserted at the end of the ninth module ACP coding sequence in recombinant DNA compounds of the invention. Recombinant DNA compounds encoding this thioesterase domain are therefore useful in constructing DNA compounds that encode a protein of the epothilone PKS, a PKS that produces an epothilone derivative, and a PKS that produces a polyketide other than epothilone or an epothilone derivative.

In one important embodiment, the present invention thus provides a hybrid PKS and the corresponding recombinant DNA compounds that encode the proteins constituting those hybrid PKS enzymes. For purposes of the present invention a hybrid PKS is a recombinant PKS that comprises all or part of one or more modules, loading domain, and thioesterase/cyclase domain of a first PKS and all or part of one or more modules, loading domain, and thioesterase/cyclase domain of a second PKS. In one preferred embodiment, the first PKS is most but not all of the epothilone PKS, and the second PKS is only a portion or all of a non-epothilone PKS. An illustrative example of such a hybrid PKS includes an epothilone PKS in which the natural loading domain has been replaced with a loading domain of another PKS. Another example of such a hybrid PKS is an epothilone PKS in which the AT domain of module four is replaced with an AT domain from a heterologous PKS that binds only methylmalonyl CoA. In another preferred embodiment, the first PKS is most but not all of a non-epothilone PKS, and the second PKS is only a portion or all of the epothilone PKS. An illustrative example of such a hybrid PKS includes an erythromycin PKS in which an AT specific for methylmalonyl CoA is replaced with an AT from the epothilone PKS specific for malonyl CoA. Another example is an erythromycin PKS that includes the MT domain of the epothilone PKS.

Those of skill in the art will recognize that all or part of either the first or second PKS in a hybrid PKS of the invention need not be isolated from a naturally occurring source. For example, only a small portion of an AT domain determines its specificity. See U.S. patent 6,221,641 ~~application Serial No. 09/346,860~~ and PCT patent application No. WO US99/15047, each of which is incorporated herein by reference. The state of the art in DNA synthesis allows the artisan to construct de novo DNA compounds of size sufficient to construct a useful portion of a PKS module or domain. For purposes of the present invention, such synthetic DNA compounds are deemed to be a portion of a PKS.

KK 7/2/02

The following Table lists references describing illustrative PKS genes and corresponding enzymes that can be utilized in the construction of the recombinant PKSs and the corresponding DNA compounds that encode them of the invention. Also presented are various references describing polyketide tailoring and modification

enzymes and corresponding genes that can be employed to make the recombinant DNA compounds of the present invention.

Avermectin

U.S. Pat. No. 5,252,474 to Merck.

MacNeil *et al.*, 1993, Industrial Microorganisms: Basic and Applied Molecular Genetics, Baltz, Hegeman, & Skatrud, eds. (ASM), pp. 245-256, A Comparison of the Genes Encoding the Polyketide Synthases for Avermectin, Erythromycin, and Nemadectin.

MacNeil *et al.*, 1992, Gene 115: 119-125, Complex Organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase.

Ikeda and Omura, 1997, Chem. Res. 97: 2599-2609, Avermectin biosynthesis.

Candicidin (FR008)

Hu *et al.*, 1994, Mol. Microbiol. 14: 163-172.

Erythromycin

PCT Pub. No. 93/13663 to Abbott.

US Pat. No. 5,824,513 to Abbott.

Donadio *et al.*, 1991, Science 252:675-9.

Cortes *et al.*, 8 Nov. 1990, Nature 348:176-8, An unusually large multifunctional polypeptide in the erythromycin producing polyketide synthase of *Saccharopolyspora erythraea*.

Glycosylation Enzymes

PCT Pat. App. Pub. No. 97/23630 to Abbott.

FK-506

Motamedi *et al.*, 1998, The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK-506, Eur. J. Biochem. 256: 528-534.

Motamedi *et al.*, 1997, Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK-506, Eur. J. Biochem. 244: 74-80.

Methyltransferase

US 5,264,355, issued 23 Nov. 1993, Methylating enzyme from *Streptomyces* MA6858. 31-O-desmethyl-FK-506 methyltransferase.

Motamedi *et al.*, 1996, Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK-506 and FK-520, J. Bacteriol. 178: 5243-5248.

KK 7/2/02

FK-520

Patent 6,150,513 issued 21 Nov. 2000

U.S. ~~patent application Serial No. 09/154,083, filed 16 Sep. 1998~~.

U.S. patent application Serial No. 09/410,551, filed 1 Oct. 1999.

Nielsen *et al.*, 1991, Biochem. 30:5789-96.

Lovastatin

U.S. Pat. No. 5,744,350 to Merck.

Narbomycin

KK 7/2/02

U.S. provisional patent application Serial No. 60/107,093, filed 5 Nov. 1998.

Nemadectin

MacNeil *et al.*, 1993, *supra*.

Niddamycin

Kakavas *et al.*, 1997, Identification and characterization of the niddamycin polyketide synthase genes from *Streptomyces caelestis*, J. Bacteriol. 179: 7515-7522.

Oleandomycin

Swan *et al.*, 1994, Characterisation of a *Streptomyces antibioticus* gene encoding a type I polyketide synthase which has an unusual coding sequence, Mol. Gen. Genet. 242: 358-362.

KK 7/2/02

U.S. provisional patent application Serial No. 60/120,254, filed 16 Feb. 1999, Serial No. 09/______, filed 28 Oct. 1999, claiming priority thereto by inventors S. Shah, M. Betlach, R. McDaniel, and L. Tang, attorney docket No. 30063-20029.00.

Olano *et al.*, 1998, Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring, Mol. Gen. Genet. 259(3): 299-308.

Picromycin

KK 7/2/02

PCT patent application No. WO US99/11814, filed 28 May 1999.

U.S. patent 6,117,659 issued 12 Sept. 2000 ~~application Serial No. 09/320,878, filed 27 May 1999.~~

U.S. patent application Serial No. 09/141,908, filed 28 Aug. 1998.

Xue *et al.*, 1998, Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the pikC-encoded cytochrome P450 in *Streptomyces venezuelae*, Chemistry & Biology 5(11): 661-667.

Xue *et al.*, Oct. 1998, A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: Architecture of metabolic diversity, Proc. Natl. Acad. Sci. USA 95: 12111-12116.

Platenolide

EP Pat. App. Pub. No. 791,656 to Lilly.

Pradimicin

PCT Pat. Pub. No. WO 98/11230 to Bristol-Myers Squibb.

Rapamycin

Schwecke *et al.*, Aug. 1995, The biosynthetic gene cluster for the polyketide rapamycin, Proc. Natl. Acad. Sci. USA 92:7839-7843.

Aparicio *et al.*, 1996, Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of the enzymatic domains in the modular polyketide synthase, Gene 169: 9-16.

Rifamycin

PCT Pat. Pub. No. WO 98/07868 to Novartis.

August *et al.*, 13 Feb. 1998, Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of Amycolatopsis mediterranei S669, Chemistry & Biology, 5(2): 69-79.

***Sorangium* PKS**

KK 7/2/02

U.S. patent 6,280,999 issued 28 Aug. 2001 ~~application Serial No. 09/144,085, filed 31 Aug. 1998.~~

Soraphen

U.S. Pat. No. 5,716,849 to Novartis.

Schupp *et al.*, 1995, J. Bacteriology 177: 3673-3679. A *Sorangium cellulosum* (Myxobacterium) Gene Cluster for the Biosynthesis of the Macrolide Antibiotic Soraphen A: Cloning, Characterization, and Homology to Polyketide Synthase Genes from Actinomycetes.

Spiramycin

U.S. Pat. No. 5,098,837 to Lilly.

Activator Gene

U.S. Pat. No. 5,514,544 to Lilly.

Tylosin

U.S. Pat. No. 5,876,991 to Lilly.

EP Pub. No. 791,655 to Lilly.

Kuhstoss *et al.*, 1996, Gene 183:231-6., Production of a novel polyketide through the construction of a hybrid polyketide synthase.

Tailoring enzymes

Merson-Davies and Cundliffe, 1994, Mol. Microbiol. 13: 349-355. Analysis of five tylosin biosynthetic genes from the tylBA region of the *Streptomyces fradiae* genome.

As the above Table illustrates, there are a wide variety of PKS genes that serve as readily available sources of DNA and sequence information for use in constructing the hybrid PKS-encoding DNA compounds of the invention. Methods for constructing hybrid PKS-encoding DNA compounds are described without reference to the epothilone PKS in U.S. Patent Nos. 5,672,491 and 5,712,146 and U.S. patent application Serial Nos. 09/073,538, filed 6 May 1998, and 09/141,908, filed 28 Aug 1998, each of which is incorporated herein by reference. Preferred PKS enzymes and coding sequences for the proteins which constitute them for purposes of isolating heterologous PKS domain coding sequences for constructing hybrid PKS enzymes of the invention are the soraphen PKS and the PKS described as a *Sorangium* PKS in the above table.

To summarize the functions of the genes cloned and sequenced in Example 1:

Gene	Protein	Modules	Domains Present
epoA	EpoA	Load	Ks^y mAT ER ACP
epoB	EpoB	1	NRPS, condensation, heterocyclization, adenylation, thiolation, PCP
epoC	EpoC	2	KS mmAT DH KR ACP
epoD	EpoD	3	KS mAT KR ACP
		4	KS mAT KR ACP
		5	KS mAT DH ER KR ACP
		6	KS mmAT DH ER KR ACP
epoE	EpoE	7	KS mmAT KR ACP
		8	KS mmAT MT DH* KR* ACP
epoF	EpoF	9	KS mAT KR DH* ACP TE

NRPS – non-ribosomal peptide synthetase; KS – ketosynthase; mAT – malonyl CoA specifying acyltransferase; mmAT – methylmalonyl CoA specifying acyltransferase; DH – dehydratase; ER – enoylreductase; KR – ketoreductase; MT – methyltransferase; TE thioesterase; * – inactive domain.

The hybrid PKS-encoding DNA compounds of the invention can be and often are hybrids of more than two PKS genes. Even where only two genes are used, there are often two or more modules in the hybrid gene in which all or part of the module is derived from a second (or third) PKS gene. Illustrative examples of recombinant epothilone derivative PKS genes of the invention, which are identified by listing the specificities of the hybrid modules (the other modules having the same specificity as the epothilone PKS), include:

(a) module 4 with methylmalonyl specific AT (mm AT) and a KR and module 2 with a malonyl specific AT (m AT) and a KR;

(b) module 4 with mM AT and a KR and module 3 with mM AT and a KR;

(c) module 4 with mM AT and a KR and module 5 with mM AT and a ER, DH, and KR;

(d) module 4 with mM AT and a KR and module 5 with mM AT and a DH and KR;

(e) module 4 with mM AT and a KR and module 5 with mM AT and a KR;

(f) module 4 with mM AT and a KR and module 5 with mM AT and an inactive KR;

(g) module 4 with mM AT and a KR and module 6 with m AT and a ER, DH, and KR;

(h) module 4 with mM AT and a KR and module 6 with m AT and a DH and KR;

(i) module 4 with mM AT and a KR and module 6 with m AT and a KR;

(j) module 4 with mM AT and a KR and module 6 with m AT and an inactive KR;

(k) module 4 with mM AT and a KR and module 7 with m AT;

(l) hybrids (c) through (f), except that module 5 has a m AT;

(m) hybrids (g) through (j) except that module 6 has a mM AT; and

(n) hybrids (a) through (m) except that module 4 has a m AT.

The above list is illustrative only and should not be construed as limiting the invention, which includes other recombinant epothilone PKS genes and enzymes with not only two hybrid modules other than those shown but also with three or more hybrid modules.

Those of skill in the art will appreciate that a hybrid PKS of the invention includes but is not limited to a PKS of any of the following types: (i) an epothilone or epothilone derivative PKS that contains a module in which at least one of the domains is from a heterologous module; (ii) an epothilone or epothilone derivative PKS that contains a module from a heterologous PKS; (iii) an epothilone or epothilone derivative PKS that contains a protein from a heterologous PKS; and (iv) combinations of the foregoing.

While an important embodiment of the present invention relates to hybrid PKS genes, the present invention also provides recombinant epothilone PKS genes in which there is no second PKS gene sequence present but which differ from the epothilone PKS gene by one or more deletions. The deletions can encompass one or more modules and/or can be limited to a partial deletion within one or more modules. When a deletion encompasses an entire module other than the NRPS module, the resulting epothilone

derivative is at least two carbons shorter than the compound produced from the PKS from which the deleted version was derived. The deletion can also encompass the NRPS module and/or the loading domain, as noted above. When a deletion is within a module, the deletion typically encompasses a KR, DH, or ER domain, or both DH and ER domains, or both KR and DH domains, or all three KR, DH, and ER domains.

The catalytic properties of the domains and modules of the epothilone PKS and of epothilone modification enzymes can also be altered by random or site specific mutagenesis of the corresponding genes. A wide variety of mutagenizing agents and methods are known in the art and are suitable for this purpose. The technique known as DNA shuffling can also be employed. See, e.g., U.S. Patent Nos. 5,830,721; 5,811,238; and 5,605,793; and references cited therein, each of which is incorporated herein by reference.

Recombinant Manipulations

To construct a hybrid PKS or epothilone derivative PKS gene of the invention, or simply to express unmodified epothilone biosynthetic genes, one can employ a technique, described in PCT Pub. No. 98/27203 and U.S. patent 6,033,883 issued 7 Mar. 2000 ~~application Serial Nos. 08/989,332~~, ~~filed 11 Dec. 1997,~~ and 60/129,731, filed 16 April 1999, each of which is incorporated herein by reference, in which the various genes of the PKS are divided into two or more, often three, segments, and each segment is placed on a separate expression vector. In this manner, the full complement of genes can be assembled and manipulated more readily for heterologous expression, and each of the segments of the gene can be altered, and various altered segments can be combined in a single host cell to provide a recombinant PKS of the invention. This technique makes more efficient the construction of large libraries of recombinant PKS genes, vectors for expressing those genes, and host cells comprising those vectors. In this and other contexts, the genes encoding the desired PKS are not only present on two or more vectors, but also can be ordered or arranged differently than in the native producer organism from which the genes were derived. Various examples of this technique as applied to the epothilone PKS are described in the Examples below. In one embodiment, the *epoA*, *epoB*, *epoC*, and *epoD* genes are present

KK 7/2/02

on a first plasmid, and the *epoE* and *epoF* and optionally either the *epoK* or the *epoK* and *epoL* genes are present on a second (or third) plasmid.

Thus, in one important embodiment, the recombinant nucleic acid compounds of the invention are expression vectors. As used herein, the term "expression vector" refers to any nucleic acid that can be introduced into a host cell or cell-free transcription and translation medium. An expression vector can be maintained stably or transiently in a cell, whether as part of the chromosomal or other DNA in the cell or in any cellular compartment, such as a replicating vector in the cytoplasm. An expression vector also comprises a gene that serves to produce RNA that is translated into a polypeptide in the cell or cell extract. Thus, the vector typically includes a promoter to enhance gene expression but alternatively may serve to incorporate the relevant coding sequence under the control of an endogenous promoter. Furthermore, expression vectors may typically contain additional functional elements, such as resistance-conferring genes to act as selectable markers and regulatory genes to enhance promoter activity.

The various components of an expression vector can vary widely, depending on the intended use of the vector. In particular, the components depend on the host cell(s) in which the vector will be used or is intended to function. Vector components for expression and maintenance of vectors in *E. coli* are widely known and commercially available, as are vector components for other commonly used organisms, such as yeast cells and *Streptomyces* cells.

In one embodiment, the vectors of the invention are used to transform *Sorangium* host cells to provide the recombinant *Sorangium* host cells of the invention. U.S. Pat. No. 5,686,295, incorporated herein by reference, describes a method for transforming *Sorangium* host cells, although other methods may also be employed. *Sorangium* is a convenient host for expressing epothilone derivatives of the invention in which the recombinant PKS that produces such derivatives is expressed from a recombinant vector in which the epothilone PKS gene promoter is positioned to drive expression of the recombinant coding sequence. The epothilone PKS gene promoter is provided in recombinant form by the present invention and is an important embodiment thereof. The promoter is contained within an ~500 nucleotide sequence between the end of the

transposon sequences and the start site of the open reading frame of the *epoA* gene. Optionally, one can include sequences from further upstream of this 500 bp region in the promoter. Those of skill in the art will recognize that, if a *Sorangium* host that produces epothilone is used as the host cell, the recombinant vector need drive expression of only a portion of the PKS containing the altered sequences. Thus, such a vector may comprise only a single altered epothilone PKS gene, with the remainder of the epothilone PKS polypeptides provided by the genes in the host cell chromosomal DNA. If the host cell naturally produces an epothilone, the epothilone derivative will thus be produced in a mixture containing the naturally occurring epothilone(s).

Those of skill will also recognize that the recombinant DNA compounds of the invention can be used to construct *Sorangium* host cells in which one or more genes involved in epothilone biosynthesis have been rendered inactive. Thus, the invention provides such *Sorangium* host cells, which may be preferred host cells for expressing epothilone derivatives of the invention so that complex mixtures of epothilones are avoided. Particularly preferred host cells of this type include those in which one or more of any of the epothilone PKS gene ORFs has been disrupted, and/or those in which any or more of the epothilone modification enzyme genes have been disrupted. Such host cells are typically constructed by a process involving homologous recombination using a vector that contains DNA homologous to the regions flanking the gene segment to be altered and positioned so that the desired homologous double crossover recombination event desired will occur.

Homologous recombination can thus be used to delete, disrupt, or alter a gene. In a preferred illustrative embodiment, the present invention provides a recombinant epothilone producing *Sorangium cellulosum* host cell in which the *epoK* gene has been deleted or disrupted by homologous recombination using a recombinant DNA vector of the invention. This host cell, unable to make the *epoK* epoxidase gene product is unable to make epothilones A and B and so is a preferred source of epothilones C and D.

Homologous recombination can also be used to alter the specificity of a PKS module by replacing coding sequences for the module or domain of a module to be altered with those specifying a module or domain of the desired specificity. In another

preferred illustrative embodiment, the present invention provides a recombinant epothilone producing *Sorangium cellulosum* host cell in which the coding sequence for the AT domain of module 4 encoded by the *epoD* gene has been altered by homologous recombination using a recombinant DNA vector of the invention to encode an AT domain that binds only methylmalonyl CoA. This host cell, unable to make epothilones A, C, and E is a preferred source of epothilones B, D, and F. The invention also provides recombinant *Sorangium* host cells in which both alterations and deletions of epothilone biosynthetic genes have been made. For example, the invention provides recombinant *Sorangium cellulosum* host cells in which both of the foregoing alteration and deletion have been made, producing a host cell that makes only epothilone D.

In similar fashion, those of skill in the art will appreciate the present invention provides a wide variety of recombinant *Sorangium cellulosum* host cells that make less complex mixtures of the epothilones than do the wild type producing cells as well as those that make one or more epothilone derivatives. Such host cells include those that make only epothilones A, C, and E; those that make only epothilones B, D, and F, those that make only epothilone D; and those that make only epothilone C.

In another preferred embodiment, the present invention provides expression vectors and recombinant *Myxococcus*, preferably *M. xanthus*, host cells containing those expression vectors that express a recombinant epothilone PKS or a PKS for an epothilone derivative. Presently, vectors that replicate extrachromosomally in *M. xanthus* are not known. There are, however, a number of phage known to integrate into *M. xanthus* chromosomal DNA, including Mx8, Mx9, Mx81, and Mx82. The integration and attachment function of these phages can be placed on plasmids to create phage-based expression vectors that integrate into the *M. xanthus* chromosomal DNA. Of these, phage Mx9 and Mx8 are preferred for purposes of the present invention. Plasmid pPLH343, described in Salmi *et al.*, Feb. 1998, Genetic determinants of immunity and integration of temperate *Myxococcus xanthus* phage Mx8, J. Bact. 180(3): 614-621, is a plasmid that replicates in *E. coli* and comprises the phage Mx8 genes that encode the attachment and integration functions.

The promoter of the epothilone PKS gene functions in *Myxococcus xanthus* host cells. Thus, in one embodiment, the present invention provides a recombinant promoter for use in recombinant host cells derived from the promoter of the *Sorangium cellulosum* epothilone PKS gene. The promoter can be used to drive expression of one or more epothilone PKS genes or another useful gene product in recombinant host cells. The invention also provides an epothilone PKS expression vector in which one or more of the epothilone PKS or epothilone modification enzyme genes are under the control of their own promoter. Another preferred promoter for use in *Myxococcus xanthus* host cells for purposes of expressing a recombinant PKS of the invention is the promoter of the pilA gene of *M. xanthus*. This promoter, as well as two *M. xanthus* strains that express high levels of gene products from genes controlled by the pilA promoter, a pilA deletion strain and a pilS deletion strain, are described in Wu and Kaiser, Dec. 1997, Regulation of expression of the pilA gene in *Myxococcus xanthus*, J. Bact. 179(24):7748-7758, incorporated herein by reference. Optionally, the invention provides recombinant *Myxococcus* host cells comprising both the pilA and pilS deletions. Another preferred promoter is the starvation dependent promoter of the sdcK gene.

Selectable markers for use in *Myxococcus xanthus* include kanamycin, tetracycline, chloramphenicol, zeocin, spectinomycin, and streptomycin resistance conferring genes. The recombinant DNA expression vectors of the invention for use in *Myxococcus* typically include such a selectable marker and may further comprise the promoter derived from an epothilone PKS or epothilone modification enzyme gene.

The present invention provides preferred expression vectors for use in preparing the recombinant *Myxococcus xanthus* expression vectors and host cells of the invention. These vectors, designated plasmids pKOS35-82.1 and pKOS35-82.2 (Figure 3), are able to replicate in *E. coli* host cells as well as integrate into the chromosomal DNA of *M. xanthus*. The vectors comprise the Mx8 attachment and integration genes as well as the pilA promoter with restriction enzyme recognition sites placed conveniently downstream. The two vectors differ from one another merely in the orientation of the pilA promoter on the vector and can be readily modified to include the epothilone PKS

and modification enzyme genes of the invention. The construction of the vectors is described in Example 2.

Especially preferred *Myxococcus* host cells of the invention are those that produce an epothilone or epothilone derivative or mixtures of epothilones or epothilone derivatives at equal to or greater than 20 mg/L, more preferably at equal to or greater than 200 mg/L, and most preferably at equal to or greater than 1 g/L. Especially preferred are *M. xanthus* host cells that produce at these levels. *M. xanthus* host cells that can be employed for purposes of the invention include the DZ1 (Campos *et al.*, 1978, J. Mol. Biol. 119: 167-178, incorporated herein by reference), the TA-producing cell line ATCC 31046, DK1219 (Hodgkin and Kaiser, 1979, Mol. Gen. Genet. 171: 177-191, incorporated herein by reference), and the DK1622 cell lines (Kaiser, 1979, Proc. Natl. Acad. Sci. USA 76: 5952-5956, incorporated herein by reference).

In another preferred embodiment, the present invention provides expression vectors and recombinant *Pseudomonas* fluorescens host cells that contain those expression vectors and express a recombinant PKS of the invention. A plasmid for use in constructing the P. fluorescens expression vectors and host cells of the invention is plasmid pRSF1010, which replicates in *E. coli* and P. fluorescens host cells (see Scholz *et al.*, 1989, Gene 75:271-8, incorporated herein by reference). Low copy number replicons and vectors can also be used. As noted above, the invention also provides the promoter of the *Sorangium cellulosum* epothilone PKS and epothilone modification enzyme genes in recombinant form. The promoter can be used to drive expression of an epothilone PKS gene or other gene in P. fluorescens host cells. Also, the promoter of the soraphen PKS genes can be used in any host cell in which a *Sorangium* promoter functions. Thus, in one embodiment, the present invention provides an epothilone PKS expression vector for use in P. fluorescens host cells.

In another preferred embodiment, the expression vectors of the invention are used to construct recombinant *Streptomyces* host cells that express a recombinant PKS of the invention. *Streptomyces* host cells useful in accordance with the invention include *S. coelicolor*, *S. lividans*, *S. venezuelae*, *S. ambofaciens*, *S. fradiae*, and the like. Preferred *Streptomyces* host cell/vector combinations of the invention include *S. coelicolor* CH999

and *S. lividans* K4-114 and K4-155 host cells, which do not produce actinorhodin, and expression vectors derived from the pRM1 and pRM5 vectors, as described in U.S. Patent No. 5,830,750 and U.S. patent 6,022,731 issued 8 Feb. 2000 ~~application Serial Nos. 08/828,898, filed 31 Mar. 1997,~~ 6,177,262 issued 23 Jan. 2001 and ~~09/181,833, filed 28 Oct. 1998~~. Especially preferred *Streptomyces* host cells of the invention are those that produce an epothilone or epothilone derivative or mixtures of epothilones or epothilone derivatives at equal to or greater than 20 mg/L, more preferably at equal to or greater than 200 mg/L, and most preferably at equal to or greater than 1 g/L. Especially preferred are *S. coelicolor* and *S. lividans* host cells that produce at these levels. Also, species of the closely related genus Saccharopolyspora can be used to produce epothilones, including but not limited to *S. erythraea*.

KK 7/2/02

The present invention provides a wide variety of expression vectors for use in *Streptomyces*. For replicating vectors, the origin of replication can be, for example and without limitation, a low copy number replicon and vectors comprising the same, such as SCP2* (see Hopwood *et al*., Genetic Manipulation of *Streptomyces*: A Laboratory manual (The John Innes Foundation, Norwich, U.K., 1985); Lydiate *et al*., 1985, Gene 35: 223-235; and Kieser and Melton, 1988, Gene 65: 83-91, each of which is incorporated herein by reference), SLP1.2 (Thompson *et al*., 1982, Gene 20: 51-62, incorporated herein by reference), and pSG5(ts) (Muth *et al*., 1989, Mol. Gen. Genet. 219: 341-348, and Bierman *et al*., 1992, Gene 116: 43-49, each of which is incorporated herein by reference), or a high copy number replicon and vectors comprising the same, such as pIJ101 and pJV1 (see Katz *et al*., 1983, J. Gen. Microbiol. 129: 2703-2714; Vara *et al*., 1989, J. Bacteriol. 171: 5782-5781; and Servin-Gonzalez, 1993, Plasmid 30: 131-140, each of which is incorporated herein by reference). High copy number vectors are generally, however, not preferred for expression of large genes or multiple genes. For non-replicating and integrating vectors and generally for any vector, it is useful to include at least an *E. coli* origin of replication, such as from pUC, p1P, p1I, and pBR. For phage based vectors, the phage phiC31 and its derivative KC515 can be employed (see Hopwood *et al*., *supra*). Also, plasmid pSET152, plasmid pSAM, plasmids pSE101 and pSE211, all of which integrate site-specifically in the chromosomal DNA of *S. lividans*, can be employed.

Typically, the expression vector will comprise one or more marker genes by which host cells containing the vector can be identified and/or selected. Useful antibiotic resistance conferring genes for use in *Streptomyces* host cells include the ermE (confers resistance to erythromycin and lincomycin), tsr (confers resistance to thiostrepton), aadA (confers resistance to spectinomycin and streptomycin), aacC4 (confers resistance to apramycin, kanamycin, gentamicin, geneticin (G418), and neomycin), hyg (confers resistance to hygromycin), and vph (confers resistance to viomycin) resistance conferring genes.

The recombinant PKS gene on the vector will be under the control of a promoter, typically with an attendant ribosome binding site sequence. A preferred promoter is the actI promoter and its attendant activator gene actII-ORF4, which is provided in the pRM1 and pRM5 expression vectors, *supra*. This promoter is activated in the stationary phase of growth when secondary metabolites are normally synthesized. Other useful *Streptomyces* promoters include without limitation those from the ermE gene and the melC1 gene, which act constitutively, and the tipA gene and the merA gene, which can be induced at any growth stage. In addition, the T7 RNA polymerase system has been transferred to *Streptomyces* and can be employed in the vectors and host cells of the invention. In this system, the coding sequence for the T7 RNA polymerase is inserted into a neutral site of the chromosome or in a vector under the control of the inducible merA promoter, and the gene of interest is placed under the control of the T7 promoter. As noted above, one or more activator genes can also be employed to enhance the activity of a promoter. Activator genes in addition to the actII-ORF4 gene discussed above include dnrI, redD, and ptpA genes (see U.S. patent 6,177,262 ~~application Serial No. 09/181,833~~, *supra*), which can be employed with their cognate promoters to drive expression of a recombinant gene of the invention.

KK 7/2/02

The present invention also provides recombinant expression vectors that drive expression of the epothilone PKS and PKS enzymes that produce epothilone or epothilone derivatives in plant cells. Such vectors are constructed in accordance with the teachings in U.S. patent 6,262,340 issued 17 Jul. 2001 ~~application Serial No. 09/114,083, filed 10 July 1998~~, and PCT patent publication No. 99/02669, each of which is incorporated herein by reference.

KK 7/2/02

Plants and plant cells expressing epothilone are disease resistant and able to resist fungal infection. For improved production of an epothilone or epothilone derivative in any heterologous host cells, including plant, *Myxococcus*, *Pseudomonas*, and *Streptomyces* host cells, one can also transform the cell to express a heterologous phosphopantetheinyl transferase. See U.S. patent application Serial No. 08/728,742, filed 11 Oct. 1996, and PCT patent publication No. 97/13845, both of which are incorporated herein by reference.

In addition to providing recombinant expression vectors that encode the epothilone or an epothilone derivative PKS, the present invention also provides, as discussed above, DNA compounds that encode epothilone modification enzyme genes. As discussed above, these gene products convert epothilones C and D to epothilones A and B, and convert epothilones A and B to epothilones E and F. The present invention also provides recombinant expression vectors and host cells transformed with those vectors that express any one or more of those genes and so produce the corresponding epothilone or epothilone derivative. In one aspect, the present invention provides the *epoK* gene in recombinant form and host cells that express the gene product thereof, which converts epothilones C and D to epothilones A and B, respectively.

In another important embodiment, and as noted above, the present invention provides vectors for disrupting the function of any one or more of the *epoL*, *epoK*, and any of the ORFs associated with the epothilone PKS gene cluster in *Sorangium* cells. The invention also provides recombinant *Sorangium* host cells lacking (or containing inactivated forms of) any one or more of these genes. These cells can be used to produce the corresponding epothilones and epothilone derivatives that result from the absence of any one or more of these genes.

The invention also provides non-*Sorangium* host cells that contain a recombinant epothilone PKS or a PKS for an epothilone derivative but do not contain (or contain non-functional forms of) any epothilone modification enzyme genes. These host cells of the invention are expected produce epothilones G and H in the absence of a dehydratase activity capable of forming the C-12-C-13 alkene of epothilones C and D. This dehydration reaction is believed to take place in the absence of the *epoL* gene product in

Streptomyces host cells. The host cells produce epothilones C and D (or the corresponding epothilone C and D derivative) when the dehydratase activity is present and the P450 epoxidase and hydroxylase (that converts epothilones A and B to epothilones E and F, respectively) genes are absent. The host cells also produce epothilones A and B (or the corresponding epothilone A and B derivatives) when the hydroxylase gene only is absent. Preferred for expression in these host cells is the recombinant epothilone PKS enzymes of the invention that contain the hybrid module 4 with an AT specific for methylmalonlyl CoA only, optionally in combination with one or more additional hybrid modules. Also preferred for expression in these host cells is the recombinant epothilone PKS enzymes of the invention that contain the hybrid module 4 with an AT specific for malonyl CoA only, optionally in combination with one or more additional hybrid modules.

The recombinant host cells of the invention can also include other genes and corresponding gene products that enhance production of a desired epothilone or epothilone derivative. As but one non-limiting example, the epothilone PKS proteins require phosphopantetheinylation of the ACP domains of the loading domain and modules 2 through 9 as well as of the PCP domain of the NRPS. Phosphopantetheinylation is mediated by enzymes that are called phosphopantetheinyl transferases (PPTases). To produce functional PKS enzyme in host cells that do not naturally express a PPTase able to act on the desired PKS enzyme or to increase amounts of functional PKS enzyme in host cells in which the PPTase is rate-limiting, one can introduce a heterologous PPTase, including but not limited to Sfp, as described in PCT Pat. Pub. Nos. 97/13845 and 98/27203, and U.S. patent application Serial Nos. 08/728,742, filed 11 Oct. 1996, and U.S. patent 6,033,883 issued 7 Mar. 2000 ~~08/989,332~~, each of which is incorporated herein by reference.

KK 7/2/02

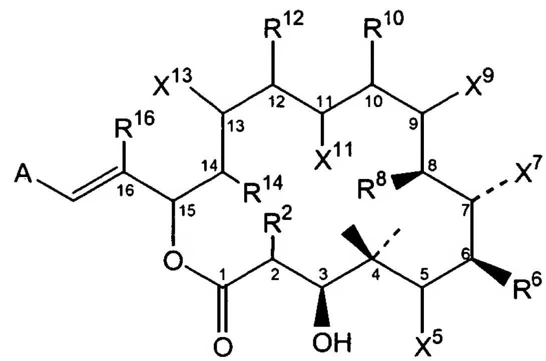
The host cells of the invention can be grown and fermented under conditions known in the art for other purposes to produce the compounds of the invention. The compounds of the invention can be isolated from the fermentation broths of these cultured cells and purified by standard procedures. Fermentation conditions for producing the compounds of the invention from *Sorangium* host cells can be based on the protocols described in PCT patent publication Nos. 93/10121, 97/19086, 98/22461, and 99/42602,

each of which is incorporated herein by reference. The novel epothilone analogs of the present invention, as well as the epothilones produced by the host cells of the invention, can be derivatized and formulated as described in PCT patent publication Nos. 93/10121, 97/19086, 98/08849, 98/22461, 98/25929, 99/01124, 99/02514, 99/07692, 99/27890, 99/39694, 99/40047, 99/42602, 99/43653, 99/43320, 99/54319, 99/54319, and 99/54330, and U.S. Patent No. 5,969,145, each of which is incorporated herein by reference.

Invention Compounds

Preferred compounds of the invention include the 14-methyl epothilone derivatives (made by utilization of the hybrid module 3 of the invention that has an AT that binds methylmalonyl CoA instead of malonyl CoA); the 8,9-dehydro epothilone derivatives (made by utilization of the hybrid module 6 of the invention that has a DH and KR instead of an ER, DH, and KR); the 10-methyl epothilone derivatives (made by utilization of the hybrid module 5 of the invention that has an AT that binds methylmalonyl CoA instead of malonyl CoA); the 9-hydroxy epothilone derivatives (made by utilization of the hybrid module 6 of the invention that has a KR instead of an ER, DH, and KR); the 8-desmethyl-14-methyl epothilone derivatives (made by utilization of the hybrid module 3 of the invention that has an AT that binds methylmalonyl CoA instead of malonyl CoA and a hybrid module 6 that binds malonyl CoA instead of methylmalonyl CoA); and the 8-desmethyl-8,9-dehydro epothilone derivatives (made by utilization of the hybrid module 6 of the invention that has a DH and KR instead of an ER, DH, and KR and an AT that specifies malonyl CoA instead of methylmalonyl CoA).

More generally, preferred epothilone derivative compounds of the invention are those that can be produced by altering the epothilone PKS genes as described herein and optionally by action of epothilone modification enzymes and/or by chemically modifying the resulting epothilones produced when those genes are expressed. Thus, the present invention provides compounds of the formula:



(1)

including the glycosylated forms thereof and stereoisomeric forms where the stereochemistry is not shown,

wherein A is a substituted or unsubstituted straight, branched chain or cyclic alkyl, alkenyl or alkynyl residue optionally containing 1-3 heteroatoms selected from O, S and N; or wherein A comprises a substituted or unsubstituted aromatic residue;

R^2 represents H,H, or H,lower alkyl, or lower alkyl,lower alkyl;

X^5 represents =O or a derivative thereof, or H,OH or H,NR_2 wherein R is H, or alkyl, or acyl or H,OCOR or H,$OCONR_2$ wherein R is H, or alkyl, or is H,H;

R^6 represents H or lower alkyl, and the remaining substituent on the corresponding carbon is H;

X^7 represents OR, NR_2, wherein R is H, or alkyl or acyl or is OCOR, or $OCONR_2$ wherein R is H or alkyl or X^7 taken together with X^9 forms a carbonate or carbamate cycle, and wherein the remaining substituent on the corresponding carbon is H;

R^8 represents H or lower alkyl and the remaining substituent on the carbon is H;

X^9 represents =O or a derivative thereof, or is H,OR or H,NR_2, wherein R is H, or alkyl or acyl or is H,OCOR or H,$OCONR_2$ wherein R is H or alkyl, or represents H,H or wherein X^9 together with X^7 or with X^{11} can form a cyclic carbonate or carbamate;

R^{10} is H,H or H,lower alkyl, or lower alkyl,lower alkyl;

X^{11} is =O or a derivative thereof, or is H,OR, or H,NR_2 wherein R is H, or alkyl or acyl or is H,OCOR or H,$OCONR_2$ wherein R is H or alkyl, or is H,H or wherein X^{11} in combination with X^9 may form a cyclic carbonate or carbamate;

R^{12} is H,H, or H,lower alkyl, or lower alkyl,lower alkyl;

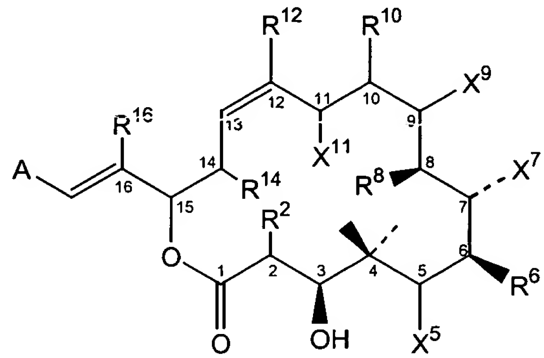
X^{13} is =O or a derivative thereof, or H,OR or H,NR_2 wherein R is H, alkyl or acyl or is H,OCOR or H,$OCONR_2$ wherein R is H or alkyl;

R^{14} is H,H, or H,lower alkyl, or lower alkyl,lower alkyl;

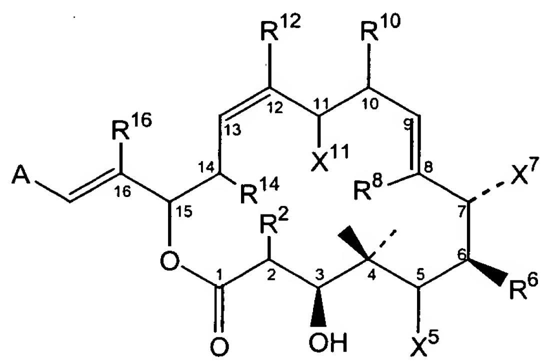
R^{16} is H or lower alkyl; and

wherein optionally H or another substituent may be removed from positions 12 and 13 and/or 8 and 9 to form a double bond, wherein said double bond may optionally be converted to an epoxide.

Particularly preferred are compounds of the formulas

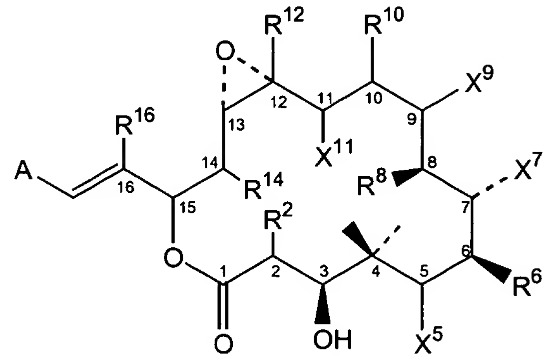


1(a),



1(b)

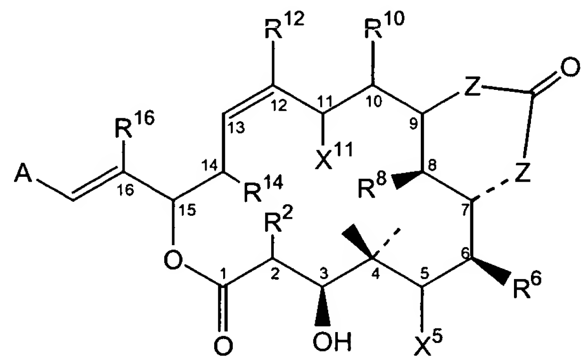
and



1(c)

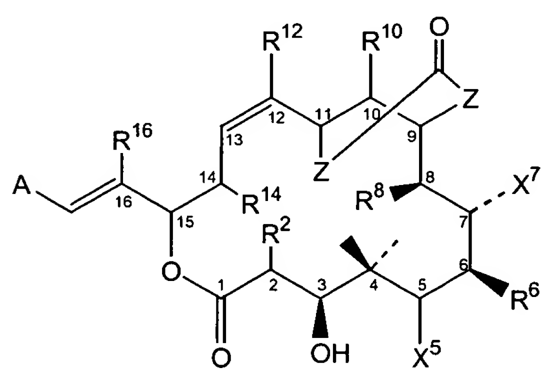
wherein the noted substituents are as defined above.

Especially preferred are compounds of the formulas



1(d)

and



1(e)

wherein both Z are O or one Z is N and the other Z is O, and the remaining substituents are as defined above.

As used herein, a substituent which "comprises an aromatic moiety" contains at least one aromatic ring, such as phenyl, pyridyl, pyrimidyl, thiophenyl, or thiazolyl. The substituent may also include fused aromatic residues such as naphthyl, indolyl, benzothiazolyl, and the like. The aromatic moiety may also be fused to a nonaromatic ring and/or may be coupled to the remainder of the compound in which it is a substituent through a nonaromatic, for example, alkylene residue. The aromatic moiety may be substituted or unsubstituted as may the remainder of the substituent.

Preferred embodiments of A include the "R" groups shown in Figure 2.

As used herein, the term alkyl refers to a C_1-C_8 saturated, straight or branched chain hydrocarbon radical derived from a hydrocarbon moiety by removal of a single hydrogen atom. Alkenyl and alkynyl refer to the corresponding unsaturated forms. Examples of alkyl include but are not limited to methyl, ethyl, propyl, isopropyl, n-butyl, tert-butyl, neopentyl, i-hexyl, n-heptyl, n-octyl. Lower alkyl (or alkenyl or alkynyl) refers to a 1-4C radical. Methyl is preferred. Acyl refers to alkylCO, alkenylCO or alkynylCO.

The terms halo and halogen as used herein refer to an atom selected from fluorine, chlorine, bromine, and iodine. The term haloalkyl as used herein denotes an alkyl group to which one, two, or three halogen atoms are attached to any one carbon and includes without limitation chloromethyl, bromoethyl, trifluoromethyl, and the like.

The term heteroaryl as used herein refers to a cyclic aromatic radical having from five to ten ring atoms of which one ring atom is selected from S, O, and N; zero, one, or two ring atoms are additional heteroatoms independently selected from S, O, and N; and the remaining ring atoms are carbon, the radical being joined to the rest of the molecule via any of the ring atoms, such as, for example, pyridyl, pyrazinyl, pyrimidinyl, pyrrolyl, pyrazolyl, imidazolyl, thiazolyl, oxazolyl, isoxazolyl, thiadiazolyl, oxadiazolyl, thiophenyl, furanyl, quinolinyl, isoquinolinyl, and the like.

The term heterocyle includes but is not limited to pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, and tetrahydrofuryl.

The term "substituted" as used herein refers to a group substituted by independent replacement of any of the hydrogen atoms thereon with, for example, Cl, Br, F, I, OH,

CN, alkyl, alkoxy, alkoxy substituted with aryl, haloalkyl, alkylthio, amino, alkylamino, dialkylamino, mercapto, nitro, carboxaldehyde, carboxy, alkoxycarbonyl, or carboxamide. Any one substituent may be an aryl, heteroaryl, or heterocycloalkyl group.

It will apparent that the nature of the substituents at positions 2, 4, 6, 8, 10, 12, 14 and 16 in formula (1) is determined at least initially by the specificity of the AT catalytic domain of modules 9, 8, 7, 6, 5, 4, 3 and 2, respectively. Because AT domains that accept malonyl CoA, methylmalonyl CoA, ethylmalonyl CoA (and in general, lower alkyl malonyl CoA), as well as hydroxymalonyl CoA, are available, one of the substituents at these positions may be H, and the other may be H, lower alkyl, especially methyl and ethyl, or OH. Further reaction at these positions, e.g., a methyl transferase reaction such as that catalyzed by module 8 of the epothilone PKS, may be used to replace H at these positions as well. Further, an H,OH embodiment may be oxidized to =O or, with the adjacent ring C, be dehydrated to form a π-bond. Both OH and =O are readily derivatized as further described below.

Thus, a wide variety of embodiments of R^2, R^6, R^8, R^{10}, R^{12}, R^{14} and R^{16} is synthetically available. The restrictions set forth with regard to embodiments of these substituents set forth in the definitions with respect to Formula (1) above reflect the information described in the SAR description in Example 8 below.

Similarly, β-carbonyl modifications (or absence of modification) can readily be controlled by modifying the epothilone PKS gene cluster to include the appropriate sequences in the corresponding positions of the epothilone gene cluster which will or will not contain active KR, DH and/or ER domains. Thus, the embodiments of X^5, X^7, X^9, X^{11} and X^{13} synthetically available are numerous, including the formation of π-bonds with the adjacent ring positions.

Positions occupied by OH are readily converted to ethers or esters by means well known in the art; protection of OH at positions not to be derivatized may be required. Further, a hydroxyl may be converted to a leaving group, such as a tosylate, and replaced by an amino or halo substituent. A wide variety of "hydroxyl derivatives" such as those discussed above is known in the art.

Similarly, ring positions which contain oxo groups may be converted to "carbonyl derivatives" such as oximes, ketals, and the like. Initial reaction products with the oxo moieties may be further reacted to obtain more complex derivatives. As described in Example 8, such derivatives may ultimately result in a cyclic substituent linking two ring positions.

The enzymes useful in modification of the polyketide initially synthesized, such as transmethylases, dehydratases, oxidases, glycosylation enzymes and the like, can be supplied endogenously by a host cell when the polyketide is synthesized intracellularly, by modifying a host to contain the recombinant materials for the production of these modifying enzymes, or can be supplied in a cell-free system, either in purified forms or as relatively crude extracts. Thus, for example, the epoxidation of the π-bond at position 12-13 may be effected using the protein product of the *epoK* gene directly *in vitro*.

The nature of A is most conveniently controlled by employing an epothilone PKS which comprises an inactivated module 1 NRPS (using a module 2 substrate) or a KS2 knockout (using a module 3 substrate) as described in Example 6, hereinbelow. Limited variation can be obtained by altering the AT catalytic specificity of the loading module; further variation is accomplished by replacing the NRPS of module 1 with an NRPS of different specificity or with a conventional PKS module. However, at present, variants are more readily prepared by feeding the synthetic module 2 substrate precursors and module 3 substrate precursors to the appropriately altered epothilone PKS as described in Example 6.

Pharmaceutical Compositions

The compounds can be readily formulated to provide the pharmaceutical compositions of the invention. The pharmaceutical compositions of the invention can be used in the form of a pharmaceutical preparation, for example, in solid, semisolid, or liquid form. This preparation will contain one or more of the compounds of the invention as an active ingredient in admixture with an organic or inorganic carrier or excipient suitable for external, enteral, or parenteral application. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers

for tablets, pellets, capsules, suppositories, pessaries, solutions, emulsions, suspensions, and any other form suitable for use.

The carriers which can be used include water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, and other carriers suitable for use in manufacturing preparations, in solid, semi-solid, or liquified form. In addition, auxiliary stabilizing, thickening, and coloring agents and perfumes may be used. For example, the compounds of the invention may be utilized with hydroxypropyl methylcellulose essentially as described in U.S. Patent No. 4,916,138, incorporated herein by reference, or with a surfactant essentially as described in EPO patent publication No. 428,169, incorporated herein by reference.

Oral dosage forms may be prepared essentially as described by Hondo *et al.*, 1987, Transplantation Proceedings XIX, Supp. 6: 17-22, incorporated herein by reference. Dosage forms for external application may be prepared essentially as described in EPO patent publication No. 423,714, incorporated herein by reference. The active compound is included in the pharmaceutical composition in an amount sufficient to produce the desired effect upon the disease process or condition.

For the treatment of conditions and diseases caused by infection, immune system disorder (or to suppress immune function), or cancer, a compound of the invention may be administered orally, topically, parenterally, by inhalation spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvant, and vehicles. The term parenteral, as used herein, includes subcutaneous injections, and intravenous, intrathecal, intramuscular, and intrasternal injection or infusion techniques.

Dosage levels of the compounds of the present invention are of the order from about 0.01 mg to about 100 mg per kilogram of body weight per day, preferably from about 0.1 mg to about 50 mg per kilogram of body weight per day. The dosage levels are useful in the treatment of the above-indicated conditions (from about 0.7 mg to about 3.5 mg per patient per day, assuming a 70 kg patient). In addition, the compounds of the

present invention may be administered on an intermittent basis, i.e., at semi-weekly, weekly, semi-monthly, or monthly intervals.

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a formulation intended for oral administration to humans may contain from 0.5 mg to 5 gm of active agent compounded with an appropriate and convenient amount of carrier material, which may vary from about 5 percent to about 95 percent of the total composition. Dosage unit forms will generally contain from about 0.5 mg to about 500 mg of active ingredient. For external administration, the compounds of the invention may be formulated within the range of, for example, 0.00001% to 60% by weight, preferably from 0.001% to 10% by weight, and most preferably from about 0.005% to 0.8% by weight.

It will be understood, however, that the specific dose level for any particular patient will depend on a variety of factors. These factors include the activity of the specific compound employed; the age, body weight, general health, sex, and diet of the subject; the time and route of administration and the rate of excretion of the drug; whether a drug combination is employed in the treatment; and the severity of the particular disease or condition for which therapy is sought.

A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the present invention and shall not be construed as being a limitation on the scope of the invention or claims.

Example 1

DNA Sequencing of Cosmid Clones and Subclones Thereof

The epothilone producing strain, *Sorangium cellulosum* SMP44, was grown on a cellulose-containing medium, see Bollag *et al.*, 1995, Cancer Research 55: 2325-2333, incorporated herein by reference, and epothilone production was confirmed by LC/MS analysis of the culture supernatant. Total DNA was prepared from this strain using the procedure described by Jaoua *et al.*, 1992, Plasmid 28: 157-165, incorporated herein by reference. To prepare a cosmid library, *S. cellulosum* genomic DNA was partially

digested with Sau3AI and ligated with BamHI-digested pSupercos (Stratagene). The DNA was packaged in lambda phage as recommended by the manufacturer and the mixture then used to infect *E. coli* XL1-Blue MR cells. This procedure yielded approximately 3,000 isolated colonies on LB-ampicillin plates. Because the size of the *S. cellulosum* genome is estimated to be circa 10^7 nucleotides, the DNA inserts present among 3000 colonies would correspond to circa 10 *S. cellulosum* genomes.

Sub B5

To screen the library, two segments of KS domains were used to design oligonucleotide primers for a PCR with *Sorangium cellulosum* genomic DNA as template. The fragment generated was then used as a probe to screen the library. This approach was chosen, because it was found, from the examination of over a dozen PKS genes, that KS domains are the most highly conserved (at the amino acid level) of all the PKS domains examined. Therefore, it was expected that the probes produced would detect not only the epothilone PKS genes but also other PKS gene clusters represented in the library. The two degenerate oligonucleotides synthesized using conserved regions within the ketosynthase (KS) domains compiled from the DEBS and soraphen PKS gene sequences were (standard nomenclature for degenerate positions is used): CTSGTSKCSSTBCACCTSGCSTGC and TGAYRTGSGCGTTSGTSCCGSWGA. A single band of ~750 bp, corresponding to the predicted size, was seen in an agarose gel after PCR employing the oligos as primers and *S. cellulosum* SMP44 genomic DNA as template. The fragment was removed from the gel and cloned in the HincII site of pUC118 (which is a derivative of pUC18 with an insert sequence for making single stranded DNA). After transformation of *E. coli*, plasmid DNA from ten independent clones was isolated and sequenced. The analysis revealed nine unique sequences that each corresponded to a common segment of KS domains in PKS genes. Of the nine, three were identical to a polyketide synthase gene cluster previously isolated from this organism and determined not to belong to the epothilone gene cluster from the analysis of the modules. The remaining six KS fragments were excised from the vector, pooled, end-labeled with ^{32}P and used as probe in hybridizations with the colonies containing the cosmid library under high stringency conditions.

The screen identified 15 cosmids that hybridized to the pooled KS probes. DNA was prepared from each cosmid, digested with NotI, separated on an agarose gel, and transferred to a nitrocellulose membrane for Southern hybridization using the pooled KS fragments as probe. The results revealed that two of the cosmids did not contain KS-hybridizing inserts, leaving 13 cosmids to analyze further. The blot was stripped of the label and re-probed, under less stringent conditions, with labeled DNA containing the sequence corresponding to the enoylreductase domain from module four of the DEBS gene cluster. Because it was anticipated that the epothilone PKS gene cluster would encode two consecutive modules that contain an ER domain, and because not all PKS gene clusters have ER domain-containing modules, hybridization with the ER probe was predicted to identify cosmids containing insert DNA from the epothilone PKS gene cluster. Two cosmids were found to hybridize strongly to the ER probe, one hybridized moderately, and a final cosmid hybridized weakly. Analysis of the restriction pattern of the NotI fragments indicated that the two cosmids that hybridized strongly with the ER probe overlapped one another. The nucleotide sequence was also obtained from the ends of each of the 13 cosmids using the T7 and T3 primer binding sites. All contained sequences that showed homology to PKS genes. Sequence from one of the cosmids that hybridized strongly to the ER probe showed homology to NRPSs and, in particular, to the adenylation domain of an NRPS. Because it was anticipated that the thiazole moiety of epothilone might be derived from the formation of an amide bond between an acetate and cysteine molecule (with a subsequent cyclization step), the presence of an NRPS domain in a cosmid that also contained ER domain(s) supported the prediction that this cosmid might contain all or part of the epothilone PKS gene cluster.

Preliminary restriction analysis of the 12 remaining cosmids suggested that three might overlap with the cosmid of interest. To verify this, oligonucleotides were synthesized for each end of the four cosmids (determined from the end sequencing described above) and used as primer sets in PCRs with each of the four cosmid DNAs. Overlap would be indicated by the appearance of a band from a non-cognate primer-template reaction. The results of this experiment verified that two of the cosmids overlapped with the cosmid containing the NRPS. Restriction mapping of the three

cosmids revealed that the cosmids did, in fact, overlap. Furthermore, because PKS sequences extended to the end of the insert in the last overlapping fragment, based on the assumption that the NRPS would map to the 5'-end of the cluster, the results also indicated that the 3' end of the gene cluster had not been isolated among the clones identified.

To isolate the remaining segment of the epothilone biosynthesis genes, a PCR fragment was generated from the cosmid containing the most 3'-terminal region of the putative gene cluster. This fragment was used as a probe to screen a newly prepared cosmid library of *Sorangium cellulosum* genomic DNA of again approximately 3000 colonies. Several hybridizing clones were identified; DNA was made from six of them. Analysis of NotI-digested fragments indicated that all contained overlapping regions. The cosmid containing the largest insert DNA that also had the shortest overlap with the cosmid used to make the probe was selected for further analysis.

Restriction maps were created for the four cosmids, as shown in Figure 1. Sequence obtained from one of the ends of cosmid pKOS35-70.8A3 showed no homology to PKS sequences or any associated modifying enzymes. Similarly, sequence from one end of cosmid pKOS35-79.85 also did not contain sequences corresponding to a PKS region. These findings supported the observation that the epothilone cluster was contained within the ~70 kb region encompassed by the four cosmid inserts.

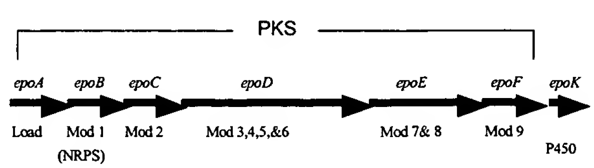
To sequence the inserts in the cosmids, each of the NotI restriction fragments from the four cosmids was cloned into the NotI site of the commercially available pBluescript plasmid. Initial sequencing was performed on the ends of each of the clones. Analysis of the sequences allowed the prediction, before having the complete sequence, that there would be 10 modules in this PKS gene cluster, a loading domain plus 9 modules.

Sequence was obtained for the complete PKS as follows. Each of the 13 non-overlapping NotI fragments was isolated and subjected to partial HinPI digestion. Fragments of ~2 to 4 kb in length were removed from an agarose gel and cloned in the AccI site of pUC118. Sufficient clones from each library of the NotI fragments were

sequenced to provide at least 4 -fold coverage of each. To sequence across each of the NotI sites, a set of oligos, one 5' and the other 3' to each NotI site, was made and used as primers in PCR amplification of a fragment that contained each NotI site. Each fragment produced in this manner was cloned and sequenced.

The nucleotide sequence was determined for a linear segment corresponding to ~72 kb. Analysis revealed a PKS gene cluster with a loading domain and nine modules. Downstream of the PKS sequence is an ORF, designated *epoK*, that shows strong homology to cytochrome P450 oxidase genes and encodes the epothilone epoxidase. The nucleotide sequence of 15 kb downstream of *epoK* has also been determined: a number of additional ORFs have been identified but an ORF that shows homology to any known dehydratase has not been identified. The *epoL* gene may encode a dehydratase activity, but this activity may instead be resident within the epothilone PKS or encoded by another gene.

The PKS genes are organized in 6 open reading frames. At the polypeptide level, the loading domain and modules 1, 2, and 9 appear on individual polypeptides; their corresponding genes are designated *epoA*, *epoB*, *epoC* and *epoF* respectively. Modules 3, 4, 5, and 6 are contained on a single polypeptide whose gene is designated *epoD*, and modules 7 and 8 are on another polypeptide whose gene is designated *epoE* . It is clear from the spacing between ORFs that *epoC*, *epoD*, *epoE* and *epoF* constitute an operon. The *epoA*, *epoB*, and *epoK* gene may be also part of the large operon, but there are spaces of approximately 100 bp between *epoB* and *epoC* and 115 bp between *epoF* and *epoK* which could contain a promoter. The present invention provides the intergenic sequences in recombinant form. At least one, but potentially more than one, promoter is used to express all of the epothilone genes. The epothilone PKS gene cluster is shown schematically below.



A detailed examination of the modules shows an organization and composition that is consistent with one able to be used for the biosynthesis of epothilone. The description that follows is at the polypeptide level. The sequence of the AT domain in the loading module and in modules 3, 4, 5, and 9 shows similarity to the consensus sequence for malonyl loading domains, consistent with the presence of an H side chain at C-14, C-12 (epothilones A and C), C-10, and C-2, respectively, as well as the loading region. The AT domains in modules 2, 6, 7, and 8 resemble the consensus sequence for methylmalonyl specifying AT domains, again consistent with the presence of methyl side chains at C-16, C-8, C-6, and C-4 respectively.

The loading module contains a KS domain in which the cysteine residue usually present at the active site is instead a tyrosine. This domain is designated as KS^y and serves as a decarboxylase, which is part of its normal function, but cannot function as a condensing enzyme. Thus, the loading domain is expected to load malonyl CoA, move it to the ACP, and decarboxylate it to yield the acetyl residue required for condensation with cysteine.

Module 1 is the non-ribosomal peptide synthetase that activates cysteine and catalyzes the condensation with acetate on the loading module. The sequence contains segments highly similar to ATP-binding and ATPase domains, required for activation of amino acids, a phosphopantotheinylation site, and an elongation domain. In database searches, module 1 shows very high similarity to a number of previously identified peptide synthetases.

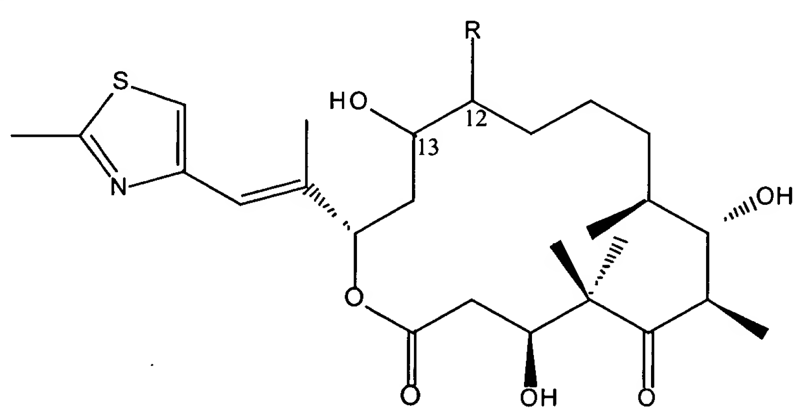
Module 2 determines the structure of epothilone at C-15 – C-17. The presence of the DH domain in module 2 yields the C-16-17 dehydro moiety in the molecule. The domains in module 3 are consistent with the structure of epothilone at C-14 and C-15; the OH that comes from the action of the KR is employed in the lactonization of the molecule.

Module 4 controls the structure at C-12 and C-13 where a double bond is found in epothilones C and D, consistent with the presence of a DH domain. Although the sequence of the AT domain appears to resemble those that specify malonate loading, it

can also load methylmalonate, thereby accounting in part for the mixture of epothilones found in the fermentation broths of the naturally producing organisms.

A significant departure from the expected array of functions was found in module 4. This module was expected to contain a DH domain, thereby directing the synthesis of epothilones C and D as the products of the PKS. Rigorous analysis revealed that the space between the AT and KR domains of module 4 was not large enough to accommodate a functional DH domain. Thus, the extent of reduction at module 4 does not proceed beyond the ketoreduction of the beta-keto formed after the condensation directed by module 4. Because the C-12,13 unsaturation has been demonstrated (epothilones C and D), there must be an additional dehydratase function that introduces the double bond, and this function is believed to be in the PKS itself or resident in an ORF in the epothilone biosynthetic gene cluster.

Thus, the action of the dehydratase could occur either during the synthesis of the polyketide or after cyclization has taken place. In the former case, the compounds produced at the end of acyl chain growth would be epothilones C and D. If the C-12,13 dehydration were a post-polyketide event, the completed acyl chain would have a hydroxyl group at C-13, as shown below. The names epothilones G and H have been assigned to the 13-hydroxy compounds produced in the absence of or prior to the action of the dehydratase.



Epothilones G (R=H) and H (R=CH_3).

Modules 5 and 6 each have the full set of reduction domains (KR, DH and ER) to yield the methylene functions at C-11 and C-9. Modules 7 and 9 have KR domains to

yield the hydroxyls at C-7 and C-3, and module 8 does not have a functional KR domain, consistent with the presence of the keto group at C-5. Module 8 also contains a methyltransferase (MT) domain that results in the presence of the geminal dimethyl function at C-4. Module 9 has a thioesterase domain that terminates polyketide synthesis and catalyzes ring closure. The genes, proteins, modules, and domains of the epothilone PKS are summarized in the Table hereinabove.

Inspection of the sequence has revealed translational coupling between *epoA* and *epoB* (loading domain and module 1) and between *epoC* and *epoD*. Very small gaps are seen between *epoD* and *epoE* and *epoE* and *epoF* but gaps exceeding 100 bp are found between *epoB* and *epoC* and *epoF* and *epoK*. These intergenic regions may contain promoters. Sequencing efforts have not revealed the presence of regulatory genes, and it is possible that epothilone synthesis is not regulated by operon specific regulation in *Sorangium cellulosum*.

sub B6

The sequence of the epothilone PKS and flanking regions has been compiled into a single contig, as shown below.

```
   1 TCGTGCGCGG GCACGTCGAG GCGTTTGCCG ACTTCGGCGG CGTCCCGCGC GTGCTGCTCT
  61 ACGACAACCT CAAGAACGCC GTCGTCGAGC GCCACGGCGA CGCGATCCGG TTCCACCCCA
 121 CGCTGCTGGC TCTGTCGGCG GATTACCGCT TCGAGCCGCG CCCCGTCGCC GTCGCCCGCG
 181 GCAACGAGAA GGGCCGCGTC GAGCGCGCCA TCCGCTACGT CCGCGAGGGC TTCTTCGAGG
 241 CCCGGGCCTA CGCCGACCTC GGAGACCTCA ACCGCCAAGC GACCGAGTGG ACCAGCTCCG
 301 CGGCGCTCGA TCGCTCCTGG GTCGAGGACC GCGCCCGCAC CGTGCGTCAG GCCTTCGACG
 361 ACGAGCGCAG CGTGCTGCTG CGACACCCTG ACACACCGTT TCCGGACCAC GAGCGCGTCG
 421 AGGTCGAGGT CGGAAAGACC CCCTACGCGC GCTTCGATCT CAACGACTAC TCGGTCCCCC
 481 ACGACCGGAC GCGCCGCACG CTGGTCGTCC TCGCCGACCT CAGTCAGGTA CGCATCGCCG
 541 ACGGCAACCA GATCGTCGCG ACCCACGTCC GTTCGTGGGA CCGCGGCCAG CAGATCGAGC
 601 AGCCCGAGCA CCTCCAGCGC CTGGTCGACG AGAAGCGCCG CGCCCGCGAG CACCGCGGCC
 661 TTGATCGCCT CGCGCGCGCC GCCCGCAGCA GCCAGGCATT CCTGCGCATC GTCGCCGAGC
 721 GCGGCGATAA CGTCGGCAGC GCGATCGCCC GGCTTCTGCA ACTGCTCGAC GCCGTGGGCG
 781 CCGCCGAGCT CGAAGAGGCC CTGGTCGAGG TGCTTGAGCG CGACACCATC CACATCGGTG
 841 CCGTCCGCCA GGTGATCGAC CGCCGCCGCT CCGAGCGCCA CCTGCCGCCT CCAGTCTCAA
 901 TCCCCGTCAC CCGCGGCGAG CACGCCGCCC TCGTCGTCAC GCCGCATTCC CTCACCACCT
 961 ACGACGCCCT GAAGAAGGAC CCGACGCCAT GACCGACCTG ACGCCCACCG AGACCAAAGA
1021 CCGGCTCAAG AGCCTCGGCC TCTTCGGCCT GCTCGCCTGC TGGGAGCAGC TCGCCGACAA
1081 GCCCTGGCTT CGCGAGGTGC TCGCCATCGA GGAGCGCGAG CGCCACAAGC GCAGCCTCGA
1141 ACGCCGCCTG AAGAACTCCC GCGTCGCCGC CTTCAAGCCC ATGACCGACT TCGACTCGTC
1201 CTGGCCCAAG AAGATCGACC GCGAGGCCGT CGACGACCTC TACGATAGCC GCTACGCGGA
1261 CCTGCTCTTC GAGGTCGTCA CCCGTCGCTA CGACGCGCAG AAGCCGCTCT TGCTCAGCAC
1321 GAACAAGGCA TTCGCCGACT GGGGCCAGGT CTTCCCGCAC GCCGCGTGCG TCGTCACGCT
1381 CGTCGACCGG CTCGTGCACC GCGCCGAGGT GATCGAGATC GAGGCCGAGA GCTACCGGCT
1441 GAAGGAAGCC AAGGAGCTCA ACGCCACCCG CACCAAGCAG CGCCGCACCA AGAAGCACTG
1501 AGCGGCATTT TCACCGGTGA ACTTCACCGA AATCCCGCGT GTTGCCGAGA TCATCTACAG
```

1561 GCGGATCGAG ACCGTGCTCA CGGCGTGGAC GACATGGCGC GGAAACGTCG TCGTAACTGC
1621 CCAGCAATGT CATGGGAATG GCCCCTTGAG GGGCTGGCCG GGGTCGACGA TATCGCGCGA
1681 TCTCCCCGTC AATTCCCGAG CGTAAAAGAA AAATTTGTCA TAGATCGTAA GCTGTGCTAG
1741 TGATCTGCCT TACGTTACGT CTTCCGCACC TCGAGCGAAT TCTCTCGGAT AACTTTCAAG
1801 TTTTCTGAGG GGGCTTGGTC TCTGGTTCCT CAGGAAGCCT GATCGGGACG AGCTAATTCC
1861 CATCCATTTT TTTGAGACTC TGCTCAAAGG GATTAGACCG AGTGAGACAG TTCTTTTGCA
1921 GTGAGCGAAG AACCTGGGGC TCGACCGGAG GACGATCGAC GTCCGCGAGC GGGTCAGCCG
1981 CTGAGGATGT GCCCGTCGTG GCGGATCGTC CCATCGAGCG CGCAGCCGAA GATCCGATTG
2041 CGATCGTCGG AGCGGGCTGC CGTCTGCCCG GTGGCGTGAT CGATCTGAGC GGGTTCTGGA
2101 CGCTCCTCGA GGGCTCGCGC GACACCGTCG GGCAAGTCCC CGCCGAACGC TGGGATGCAG
2161 CAGCGTGGTT TGATCCCGAC CTCGATGCCC CGGGGAAGAC GCCCGTTACG CGCGCATCTT
2221 TCCTGAGCGA CGTAGCCTGC TTCGACGCCT CCTTCTTCGG CATCTCGCCT CGCGAAGCGC
2281 TGCGGATGGA CCCTGCACAT CGACTCTTGC TGGAGGTGTG CTGGGAGGCG CTGGAGAACG
2341 CCGCGATCGC TCCATCGGCG CTCGTCGGTA CGGAAACGGG AGTGTTCATC GGGATCGGCC
2401 CGTCCGAATA TGAGGCCGCG CTGCCGCGAG CGACGGCGTC CGCAGAGATC GACGCTCATG
2461 GCGGGCTGGG GACGATGCCC AGCGTCGGAG CGGGCCGAAT CTCGTATGTC CTCGGGCTGC
2521 GAGGGCCGTG TGTCGCGGTG GATACGGCCT ATTCGTCCTC GCTCGTGGCC GTTCATCTGG
2581 CCTGTCAGAG CTTGCGCTCC GGGGAATGCT CCACGGCCCT GGCTGGTGGG GTATCGCTGA
2641 TGTTGTCGCC GAGCACCCTC GTGTGGCTCT CGAAGACCCG CGCGCTGGCC ACGGACGGTC
2701 GCTGCAAGGC GTTTTCGGCG GAGGCCGATG GGTTCGGACG AGGCGAAGGG TGCGCCGTCG
2761 TGGTCCTCAA GCGGCTCAGT GGAGCCCGCG CGGACGGCGA CCGGATATTG GCGGTGATTC
2821 GAGGATCCGC GATCAATCAC GACGGAGCGA GCAGCGGTCT GACCGTGCCG AACGGGAGCT
2881 CCCAAGAAAT CGTGCTGAAA CGGGCCCTGG CGGACGCAGG CTGCGCCGCG TCTTCGGTGG
2941 GTTATGTCGA GGCACACGGC ACGGGCACGA CGCTTGGTGA CCCCATCGAA ATCCAAGCTC
3001 TGAATGCGGT ATACGGCCTC GGGCGAGACG TCGCCACGCC GCTGCTGATC GGGTCGGTGA
3061 AGACCAACCT TGGCCATCCT GAGTATGCGT CGGGGATCAC TGGGCTGCTG AAGGTCGTCT
3121 TGTCCCTTCA GCACGGGCAG ATTCCTGCGC ACCTCCACGC GCAGGCGCTG AACCCCCGGA
3181 TCTCATGGGG TGATCTTCGG CTGACCGTCA CGCGCGCCCG GACACCGTGG CCGGACTGGA
3241 ATACGCCGCG ACGGGCGGGG GTGAGCTCGT TCGGCATGAG CGGGACCAAC GCGCACGTGG
3301 TGCTGGAAGA GGCGCCGGCG GCGACGTGCA CACCGCCGGC GCCGGAGCGG CCGGCAGAGC
3361 TGCTGGTGCT GTCGGCAAGG ACCGCGGCAG CCTTGGATGC ACACGCGGCG CGGCTGCGCG
3421 ACCATCTGGA GACCTACCCT TCGCAGTGTC TGGGCGATGT GGCGTTCAGT CTGGCGACGA
3481 CGCGCAGCGC GATGGAGCAC CGGCTCGCGG TGGCGGCGAC GTCGAGCGAG GGGCTGCGGG
3541 CAGCCCTGGA CGCTGCGGCG CAGGGACAGA CGCCGCCCGG TGTGGTGCGC GGTATCGCCG
3601 ATTCCTCACG CGGCAAGCTC GCCTTTCTCT TCACCGGACA GGGGGCGCAG ACGCTGGGCA
3661 TGGGCCGTGG GCTGTATGAT GTATGGCCCG CGTTCCGCGA GGCGTTCGAC CTGTGCGTGA
3721 GGCTGTTCAA CCAGGAGCTC GACCGGCCGC TCCGCGAGGT GATGTGGGCC GAACCGGCCA
3781 GCGTCGACGC CGCGCTGCTC GACCAGACAG CCTTTACCCA GCCGGCGCTG TTCACCTTCG
3841 AGTATGCGCT CGCCGCGCTG TGGCGGTCGT GGGGCGTAGA GCCGGAGTTG GTCGCTGGCC
3901 ATAGCATCGG TGAGCTGGTG GCTGCCTGCG TGGCGGGCGT GTTCTCGCTT GAGGACGCGG
3961 TGTTCCTGGT GGCTGCGCGC GGGCGCCTGA TGCAGGCGCT GCCGGCCGGC GGGGCGATGG
4021 TGTCGATCGC GGCGCCGGAG GCCGATGTGG CTGCTGCGGT GGCGCCGCAC GCAGCGTCGG
4081 TGTCGATCGC CGCGGTCAAC GGTCCGGACC AGGTGGTCAT CGCGGGCGCC GGGCAACCCG
4141 TGCATGCGAT CGCGGCGGCG ATGGCCGCGC GCGGGGCGCG AACCAAGGCG CTCCACGTCT
4201 CGCATGCGTT CCACTCACCG CTCATGGCCC CGATGCTGGA GGCGTTCGGG CGTGTGGCCG
4261 AGTCGGTGAG CTACCGGCGG CCGTCGATCG TCCTGGTCAG CAATCTGAGC GGGAAGGCTG
4321 GCACAGACGA GGTGAGCTCG CCGGGCTATT GGGTGCGCCA CGCGCGAGAG GTGGTGCGCT
4381 TCGCGGATGG AGTGAAGGCG CTGCACGCGG CCGGTGCGGG CACCTTCGTC GAGGTCGGTC
4441 CGAAATCGAC GCTGCTCGGC CTGGTGCCTG CCTGCCTGCC GGACGCCCGG CCGGCGCTGC
4501 TCGCATCGTC GCGCGCTGGG CGTGACGAGC CAGCGACCGT GCTCGAGGCG CTCGGCGGGC
4561 TCTGGGCCGT CGGTGGCCTG GTCTCCTGGG CCGGCCTCTT CCCCTCAGGG GGGCGGCGGG
4621 TGCCGCTGCC CACGTACCCT TGGCAGCGCG AGCGCTACTG GATCGACACG AAAGCCGACG
4681 ACGCGGCGCG TGGCGACCGC CGTGCTCCGG GAGCGGGTCA CGACGAGGTC GAGAAGGGGG
4741 GCGCGGTGCG CGGCGGCGAC CGGCGCAGCG CTCGGCTCGA CCATCCGCCG CCCGAGAGCG

```
4801 GACGCCGGGA GAAGGTCGAG GCCGCCGGCG ACCGTCCGTT CCGGCTCGAG ATCGATGAGC
4861 CAGGCGTGCT CGATCGCCTG GTGCTTCGGG TCACGGAGCG GCGCGCCCCT GGTCTTGGCG
4921 AGGTCGAGAT CGCCGTCGAC GCGGCGGGGC TCAGCTTCAA TGATGTCCAG CTCGCGCTGG
4981 GCATGGTGCC CGACGACCTG CCGGGAAAGC CCAACCCTCC GCTGCTGCTC GGAGGCGAGT
5041 GCGCCGGGCG CATCGTCGCC GTGGGCGAGG GCGTGAACGG CCTTGTGGTG GGCCAACCGG
5101 TCATCGCCCT TTCGGCGGGA GCGTTTGCTA CCCACGTCAC CACGTCGGCT GCGCTGGTGC
5161 TGCCTCGGCC TCAGGCGCTC TCGGCGACCG AGGCGGCCGC CATGCCCGTC GCGTACCTGA
5221 CGGCATGGTA CGCGCTCGAC GGAATAGCCC GCCTTCAGCC GGGGGAGCGG GTGCTGATCC
5281 ACGCGGCGAC CGGCGGGGTC GGTCTCGCCG CGGTGCAGTG GGCGCAGCAC GTGGGAGCCG
5341 AGGTCCATGC GACGGCCGGC ACGCCCGAGA AGCGCGCCTA CCTGGAGTCG CTGGGCGTGC
5401 GGTATGTGAG CGATTCCCGC TCGGACCGGT TCGTCGCCGA CGTGCGCGCG TGGACGGGCG
5461 GCGAGGGAGT AGACGTCGTG CTCAACTCGC TTTCGGGCGA GCTGATCGAC AAGAGTTTCA
5521 ATCTCCTGCG ATCGCACGGC CGGTTTGTGG AGCTCGGCAA GCGCGACTGT TACGCGGATA
5581 ACCAGCTCGG GCTGCGGCCG TTCCTGCGCA ATCTCTCCTT CTCGCTGGTG GATCTCCGGG
5641 GGATGATGCT CGAGCGGCCG GCGCGGGTCC GTGCGCTCTT CGAGGAGCTC CTCGGCCTGA
5701 TCGCGGCAGG CGTGTTCACC CCTCCCCCCA TCGCGACGCT CCCGATCGCT CGTGTCGCCG
5761 ATGCGTTCCG GAGCATGGCG CAGGCGCAGC ATCTTGGGAA GCTCGTACTC ACGCTGGGTG
5821 ACCCGGAGGT CCAGATCCGT ATTCCGACCC ACGCAGGCGC CGGCCCGTCC ACCGGGGATC
5881 GGGATCTGCT CGACAGGCTC GCGTCAGCTG CGCCGGCCGC GCGCGCGGCG GCGCTGGAGG
5941 CGTTCCTCCG TACGCAGGTC TCGCAGGTGC TGCGCACGCC CGAAATCAAG GTCGGCGCGG
6001 AGGCGCTGTT CACCCGCCTC GGCATGGACT CGCTCATGGC CGTGGAGCTG CGCAATCGTA
6061 TCGAGGCGAG CCTCAAGCTG AAGCTGTCGA CGACGTTCCT GTCCACGTCC CCCAATATCG
6121 CCTTGTTGAC CCAAAACCTG TTGGATGCTC TCGCCACAGC TCTCTCCTTG GAGCGGGTGG
6181 CGGCGGAGAA CCTACGGGCA GGCGTGCAAA GCGACTTCGT CTCATCGGGC GCAGATCAAG
6241 ACTGGGAAAT CATTGCCCTA TGACGATCAA TCAGCTTCTG AACGAGCTCG AGCACCAGGG
6301 TGTCAAGCTG GCGGCCGATG GGAGCGCCT CCAGATACAG GCCCCCAAGA ACGCCCTGAA
6361 CCCGAACCTG CTCGCTCGAA TCTCCGAGCA CAAAAGCACG ATCCTGACGA TGCTCCGTCA
6421 GAGACTCCCC GCAGAGTCCA TCGTGCCCGC CCCAGCCGAG CGGCACGTTC CGTTTCCTCT
6481 CACAGACATC CAAGGATCCT ACTGGCTGGG TCGGACAGGA GCGTTTACGG TCCCCAGCGG
6541 GATCCACGCC TATCGCGAAT ACGACTGTAC GGATCTCGAC GTGGCGAGGC TGAGCCGCGC
6601 CTTTCGGAAA GTCGTCGCGC GGCACGACAT GCTTCGGGCC CACACGCTGC CCGACATGAT
6661 GCAGGTGATC GAGCCTAAAG TCGACGCCGA CATCGAGATC ATCGATCTGC GCGGGCTCGA
6721 CCGGAGCACA CGGGAAGCGA GGCTCGTATC GTTGCGAGAT GCGATGTCGC ACCGCATCTA
6781 TGACACCGAG CGCCCTCCGC TCTATCACGT CGTCGCCGTT CGGCTGGACG AGCAGCAAAC
6841 CCGTCTCGTG CTCAGTATCG ATCTCATTAA CGTTGACCTA GGCAGCCTGT CCATCATCTT
6901 CAAGGATTGG CTCAGCTTCT ACGAAGATCC CGAGACCTCT CTCCCTGTCC TGGAGCTCTC
6961 GTACCGCGAC TATGTGCTCG CGCTGGAGTC TCGCAAGAAG TCTGAGGCGC ATCAACGATC
7021 GATGGATTAC TGGAAGCGGC GCGTCGCCGA GCTCCCACCT CCGCCGATGC TTCCGATGAA
7081 GGCCGATCCA TCTACCCTGA GGGAGATCCG CTTCCGGCAC ACGGAGCAAT GGCTGCCGTC
7141 GGACTCCTGG AGTCGATTGA AGCAGCGTGT CGGGGAGCGC GGGCTGACCC CGACGGGCGT
7201 CATTCTGGCT GCATTTTCCG AGGTGATCGG GCGCTGGAGC GCGAGCCCCC GGTTTACGCT
7261 CAACATAACG CTCTTCAACC GGCTCCCCGT CCATCCGCGC GTGAACGATA TCACCGGGGA
7321 CTTCACGTCG ATGGTCCTCC TGGACATCGA CACCACTCGC GACAAGAGCT TCGAACAGCG
7381 CGCTAAGCGT ATTCAAGAGC AGCTGTGGGA AGCGATGGAT CACTGCGACG TAAGCGGTAT
7441 CGAGGTCCAG CGAGAGGCCG CCCGGGTCCT GGGGATCCAA CGAGGCGCAT TGTTCCCCGT
7501 GGTGCTCACG AGCGCGCTCA ACCAGCAAGT CGTTGGTGTC ACCTCGCTGC AGAGGCTCGG
7561 CACTCCGGTG TACACCAGCA CGCAGACTCC TCAGCTGCTG CTGGATCATC AGCTCTACGA
7621 GCACGATGGG GACCTCGTCC TCGCGTGGGA CATCGTCGAC GGAGTGTTCC CGCCCGACCT
7681 TCTGGACGAC ATGCTCGAAG CGTACGTCGC TTTTCTCCGG CGGCTCACTG AGGAACCATG
7741 GAGTGAACAG ATGCGCTGTT CGCTTCCGCC TGCCCAGCTA GAAGCGCGGG CGAGCGCAAA
7801 CGAGACCAAC TCGCTGCTGA GCGAGCATAC GCTGCACGGC CTGTTCGCGG CGCGGGTCGA
7861 GCAGCTGCCT ATGCAGCTCG CCGTGGTGTC GGCGCGCAAG ACGCTCACGT ACGAAGAGCT
7921 TTCGCGCCGT TCGCGGCGAC TTGGCGCGCG GCTGCGCGAG CAGGGGGCAC GCCCGAACAC
7981 ATTGGTCGCG GTGGTGATGG AGAAAGGCTG GGAGCAGGTT GTCGCGGTTC TCGCGGTGCT
```

```
 8041 CGAGTCAGGC GCGGCCTACG TGCCGATCGA TGCCGACCTA CCGGCGGAGC GTATCCACTA
 8101 CCTCCTCGAT CATGGTGAGG TAAAGCTCGT GCTGACGCAG CCATGGCTGG ATGGCAAACT
 8161 GTCATGGCCG CCGGGGATCC AGCGGCTGCT CGTGAGCGAT GCCGGCGTCG AAGGCGACGG
 8221 CGACCAGCTT CCGATGATGC CCATTCAGAC ACCTTCGGAT CTCGCGTATG TCATCTACAC
 8281 CTCGGGATCC ACAGGGTTGC CCAAGGGGGT GATGATCGAT CATCGGGGTG CCGTCAACAC
 8341 CATCCTGGAC ATCAACGAGC GCTTCGAAAT AGGGCCCGGA GACAGAGTGC TGGCGCTCTC
 8401 CTCGCTGAGC TTCGATCTCT CGGTCTACGA TGTGTTCGGG ATCCTGGCGG CGGGCGGTAC
 8461 GATCGTGGTG CCGGACGCGT CCAAGCTGCG CGATCCGGCG CATTGGGCAG CGTTGATCGA
 8521 ACGAGAGAAG GTGACGGTGT GGAACTCGGT GCCGGCGCTG ATGCGGATGC TCGTCGAGCA
 8581 TTCCGAGGGT CGCCCCGATT CGCTCGCTAG GTCTCTGCGG CTTTCGCTGC TGAGCGGCGA
 8641 CTGGATCCCG GTGGGCCTGC CTGGCGAGCT CCAGGCCATC AGGCCCGGCG TGTCGGTGAT
 8701 CAGCCTGGGC GGGGCCACCG AAGCGTCGAT CTGGTCCATC GGGTACCCCG TGAGGAACGT
 8761 CGATCCATCG TGGGCGAGCA TCCCCTACGG CCGTCCGCTG CGCAACCAGA CGTTCCACGT
 8821 GCTCGATGAG GCGCTCGAAC CGCGCCCGGT CTGGGTTCCG GGCAACTCT ACATTGGCGG
 8881 GGTCGGACTG GCACTGGGCT ACTGGCGCGA TGAAGAGAAG ACGCGCAACA GCTTCCTCGT
 8941 GCACCCCGAG ACCGGGGAGC GCCTCTACAA GACCGGCGAT CTGGGCCGCT ACCTGCCCGA
 9001 TGGAAACATC GAGTTCATGG GGCGGGAGGA CAACCAAATC AAGCTTCGCG GATACCGCGT
 9061 TGAGCTCGGG GAAATCGAGG AAACGCTCAA GTCGCATCCG AACGTACGCG ACGCGGTGAT
 9121 TGTGCCCGTC GGGAACGACG CGGCGAACAA GCTCCTTCTA GCCTATGTGG TCCCGGAAGG
 9181 CACACGGAGA CGCGCTGCCG AGCAGGACGC GAGCCTCAAG ACCGAGCGGG TCGACGCGAG
 9241 AGCACACGCC GCCAAAGCGG ACGGATTGAG CGACGGCGAG AGGGTGCAGT TCAAGCTCGC
 9301 TCGACACGGA CTCCGGAGGG ATCTGGACGG AAAGCCCGTC GTCGATCTGA CCGGGCTGGT
 9361 TCCGCGGGAG GCGGGGCTGG ACGTCTACGC GCGTCGCCGT AGCGTCCGAA CGTTCCTCGA
 9421 GGCCCCGATT CCATTTGTTG AATTCGGCCG ATTCCTGAGC TGCCTGAGCA GCGTGGAGCC
 9481 CGACGGCGCG GCCCTTCCCA AATTCCGTTA TCCATCGGCT GGCAGCACGT ACCCGGTGCA
 9541 AACCTACGCG TACGCCAAAT CCGGCCGCAT CGAGGGCGTG GACGAGGGCT TCTATTATTA
 9601 CCACCCGTTC GAGCACCGTT TGCTGAAGGT CTCCGATCAC GGGATCGAGC GCGGAGCGCA
 9661 CGTTCCGCAA AACTTCGACG TGTTCGATGA AGCGGCGTTC GGCCTCCTGT TCGTGGGCAG
 9721 GATCGATGCC ATCGAGTCGC TGTATGGATC GTTGTCACGA GAATTCTGCC TGCTGGAGGC
 9781 CGGATATATG GCGCAGCTCC TGATGGAGCA GGCGCCTTCC TGCAACATCG GCGTCTGTCC
 9841 GGTGGGTCAA TTCGATTTTG AACAGGTTCG GCCGGTTCTC GACCTGCGGC ATTCGGACGT
 9901 TTACGTGCAC GGCATGCTGG GCGGGCGGGT AGACCCGCGG CAGTTCCAGG TCTGTACGCT
 9961 CGGTCAGGAT TCCTCACCGA GGCGCGCCAC GACGCGCGGC GCCCCTCCCG GCCGCGATCA
10021 GCACTTCGCC GATATCCTTC GCGACTTCTT GAGGACCAAA CTACCCGAGT ACATGGTGCC
10081 TACAGTCTTC GTGGAGCTCG ATGCGTTGCC GCTGACGTCC AACGGCAAGG TCGATCGTAA
10141 GGCCCTGCGC GAGCGGAAGG ATACCTCGTC GCCGCGGCAT TCGGGGCACA CGGCGCCACG
10201 GGACGCCTTG GAGGAGATCC TCGTTGCGGT CGTACGGGAG GTGCTCGGGC TGGAGGTGGT
10261 TGGGCTCCAG CAGAGCTTCG TCGATCTTGG TGCGACATCG ATTCACATCG TTCGCATGAG
10321 GAGTCTGTTG CAGAAGAGGC TGGATAGGGA GATCGCCATC ACCGAGTTGT TCCAGTACCC
10381 GAACCTCGGC TCGCTGGCGT CCGGTTTGCG CCGAGACTCG AAAGATCTAG AGCAGCGGCC
10441 GAACATGCAG GACCGAGTGG AGGCTCGGCG CAAGGGCAGG AGACGTAGCT AAGAGCGCCG
10501 AACAAAACCA GGCCGAGCGG GCCAATGAAC CGCAAGCCCG CCTGCGTCAC CCTGGGACTC
10561 ATCTGATCTG ATCGCGGGTA CGCGTCGCGG GTGTGCGCGT TGAGCCGTGT TGCTCGAACG
10621 CTGAGGAACG GTGAGCTCAT GGAAGAACAA GAGTCCTCCG CTATCGCAGT CATCGGCATG
10681 TCGGGCCGTT TTCCGGGGGC GCGGGATCTG GACGAATTCT GGAGGAACCT TCGAGACGGC
10741 ACGGAGGCCG TGCAGCGCTT CTCCGAGCAG GAGCTCGCGG CGTCCGGAGT CGACCCAGCG
10801 CTGGTGCTGG ACCCGAACTA CGTCCGGGCG GGCAGCGTGC TGGAAGATGT CGACCGGTTC
10861 GACGCTGCTT TCTTCGGCAT CAGCCCGCGC GAGGCAGAGC TCATGGATCC GCAGCACCGC
10921 ATCTTCATGG AATGCGCCTG GGAGGCGCTG GAGAACGCCG GATACGACCC GACAGCCTAC
10981 GAGGGCTCTA TCGGCGTGTA CGCCGGCGCC AACATGAGCT CGTACTTGAC GTCGAACCTC
11041 CACGAGCACC CAGCGATGAT GCGGTGGCCC GGCTGGTTTC AGACGTTGAT CGGCAACGAC
11101 AAGGATTACC TCGCGACCCA CGTCTCCTAC AGGCTGAATC TGAGAGGGCC GAGCATCTCC
11161 GTTCAAACTG CCTGCTCTAC CTCGCTCGTG GCGGTTCACT TGGCGTGCAT GAGCCTCCTG
11221 GACCGCGAGT GCGACATGGC GCTGGCCGGC GGGATTACCG TCCGGATCCC CCATCGAGCC
```

11281 GGCTATGTAT ATGCTGAGGG GGGCATCTTC TCTCCCGACG GCCATTGCCG GGCCTTCGAC
11341 GCCAAGGCGA ACGGCACGAT CATGGGCAAC GGCTGCGGGG TTGTCCTCCT GAAGCCGCTG
11401 GACCGGGCGC TCTCCGATGG TGATCCCGTC CGCGCGGTCA TCCTTGGGTC TGCCACAAAC
11461 AACGACGGAG CGAGGAAGAT CGGGTTCACT GCGCCCAGTG AGGTGGGCCA GGCGCAAGCG
11521 ATCATGGAGG CGCTGGCGCT GGCAGGGGTC GAGGCCCGGT CCATCCAATA CATCGAGACC
11581 CACGGGACCG GCACGCTGCT CGGAGACGCC ATCGAGACGG CGGCGTTGCG GCGGGTGTTC
11641 GATCGCGACG CTTCGACCCG GAGGTCTTGC GCGATCGGCT CCGTGAAGAC CGGCATCGGA
11701 CACCTCGAAT CGGCGGCTGG CATCGCCGGT TTGATCAAGA CGGTCTTGGC GCTGGAGCAC
11761 CGGCAGCTGC CGCCCAGCCT GAACTTCGAG TCTCCTAACC CATCGATCGA TTTCGCGAGC
11821 AGCCCGTTCT ACGTCAATAC CTCTCTTAAG GATTGGAATA CCGGCTCGAC TCCGCGGCGG
11881 GCCGGCGTCA GCTCGTTCGG GATCGGCGGC ACCAACGCCC ATGTCGTGCT GGAGGAAGCA
11941 CCCGCGGCGA AGCTTCCAGC CGCGGCGCCG GCGCGCTCTG CCGAGCTCTT CGTCGTCTCG
12001 GCCAAGAGCG CAGCGGCGCT GGATGCCGCG GCGGCACGGC TACGAGATCA TCTGCAGGCG
12061 CACCAGGGGC TTTCGTTGGG CGACGTCGCC TTCAGCCTGG CGACGACGCG CAGTCCCATG
12121 GAGCACCGGC TCGCGATGGC GGCACCGTCG CGCGAGGCGT TGCGAGAGGG GCTCGACGCA
12181 GCGGCGCGAG GCCAGACCCC GCCGGGCGCC GTGCGTGGCC GCTGCTCCCC AGGCAACGTG
12241 CCGAAGGTGG TCTTCGTCTT TCCCGGCCAG GGCTCTCAGT GGGTCGGTAT GGGCCGTCAG
12301 CTCCTGGCTG AGGAACCCGT CTTCCACGCG GCGCTTTCGG CGTGCGACCG GGCCATCCAG
12361 GCCGAAGCTG GTTGGTCGCT GCTCGCCGAG CTCGCCGCCG ACGAAGGGTC GTCCCAGATC
12421 GAGCGCATCG ACGTGGTGCA GCCGGTGCTG TTCGCGCTCG CGGTGGCATT TGCGGCGCTG
12481 TGGCGGTCGT GGGGTGTCGG GCCCGACGTC GTGATCGGCC ACAGCATGGG CGAGGTAGCC
12541 GCCGCGCATG TGGCCGGGGC GCTGTCGCTC GAGGATGCGG TGGCGATCAT CTGCCGGCGC
12601 AGCCGGCTGC TCCGGCGCAT CAGCGGTCAG GGCGAGATGG CGGTGACCGA GCTGTCGCTG
12661 GCCGAGGCCG AGGCAGCGCT CCGAGGCTAC GAGGATCGGG TGAGCGTGGC CGTGAGCAAC
12721 AGCCCGCGCT CGACGGTGCT CTCGGGCGAG CCGGCAGCGA TCGGCGAGGT GCTGTCGTCC
12781 CTGAACGCGA AGGGGGTGTT CTGCCGTCGG GTGAAGGTGG ATGTCGCCAG CCACAGCCCG
12841 CAGGTCGACC CGCTGCGCGA GGACCTCTTG GCAGCGCTGG GCGGGCTCCG GCCGCGTGCG
12901 GCTGCGGTGC CGATGCGCTC GACGGTGACG GGCGCCATGG TAGCGGGCCC GGAGCTCGGA
12961 GCGAATTACT GGATGAACAA TCTCAGGCAG CCTGTGCGCT TCGCCGAGGT AGTCCAGGCG
13021 CAGCTCCAAG GCGGCCACGG TCTGTTCGTG GAGATGAGCC CGCATCCGAT CCTAACGACT
13081 TCGGTCGAGG AGATGCGGCG CGCGGCCCAG CGGGCGGGCG CAGCGGTGGG CTCGCTGCGG
13141 CGAGGGCAGG ACGAGCGCCC GGCGATGCTG GAGGCGCTGG GCGCGCTGTG GGCGCAGGGC
13201 TACCCTGTAC CCTGGGGGCG GCTGTTTCCC GCGGGGGGGC GGCGGGTACC GCTGCCGACC
13261 TATCCCTGGC AGCGCGAGCG GTACTGGATC GAAGCGCCGG CCAAGAGCGC CGCGGGCGAT
13321 CGCCGCGGCG TGCGTGCGGG CGGTCACCCG CTCCTCGGTG AAATGCAGAC CCTATCAACC
13381 CAGACGAGCA CGCGGCTGTG GGAGACGACG CTGGATCTCA AGCGGCTGCC GTGGCTCGGC
13441 GACCACCGGG TGCAGGGAGC GGTCGTGTTT CCGGGCGCGG CGTACCTGGA GATGGCGATT
13501 TCGTCGGGGG CCGAGGCTTT GGGCGATGGC CCATTGCAGA TAACCGACGT GGTGCTCGCC
13561 GAGGCGCTGG CCTTCGCGGG CGACGCGGCG GTGTTGGTCC AGGTGGTGAC GACGGAGCAG
13621 CCGTCGGGAC GGCTGCAGTT CCAGATCGCG AGCCGGGCGC CGGGCGCTGG CCACGCGTCC
13681 TTCCGGGTCC ACGCTCGCGG CGCGTTGCTC CGAGTGGAGC GCACCGAGGT CCCGGCTGGG
13741 CTTACGCTTT CCGCCGTGCG CGCACGGCTC CAGGCCAGCA TGCCCGCCGC GGCCACCTAC
13801 GCGGAGCTGA CCGAGATGGG GCTGCAGTAC GGCCCTGCCT TCCAGGGGAT TGCTGAGCTA
13861 TGGCGCGGTG AGGGCGAGGC GCTGGGACGG GTACGCCTGC CCGACGCGGC CGGCTCGGCA
13921 GCGGAGTATC GGTTGCATCC TGCGCTGCTG GACGCGTGCT TCCAGGTCGT CGGCAGCCTC
13981 TTCGCCGGCG GTGGCGAGGC GACGCCGTGG GTGCCCGTGG AAGTGGGCTC GCTGCGGCTC
14041 TTGCAGCGGC CTTCGGGGGA GCTGTGGTGC CATGCGCGCG TCGTGAACCA CGGGCGCCAA
14101 ACCCCCGATC GGCAGGGCGC CGACTTTTGG GTGGTCGACA GCTCGGGTGC AGTGGTCGCC
14161 GAAGTCAGCG GGCTCGTGGC GCAGCGGCTT CCGGGAGGGG TGCGCCGGCG CGAAGAAGAC
14221 GATTGGTTCC TGGAGCTCGA GTGGGAACCC GCAGCGGTCG GCACAGCCAA GGTCAACGCG
14281 GGCCGGTGGC TGCTCCTCGG CGGCGGCGGT GGGCTCGGCG CCGCGTTGCG CTCGATGCTG
14341 GAGGCCGGCG GCCATGCCGT CGTCCATGCG GCAGAGAGCA ACACGAGCGC TGCCGGCGTA
14401 CGCGCGCTCC TGGCAAAGGC CTTTGACGGC CAGGCTCCGA CGGCGGTGGT GCACCTCGGC
14461 AGCCTCGATG GGGGTGGCGA GCTCGACCCA GGGCTCGGGG CGCAAGGCGC ATTGGACGCG

14521 CCCCGGAGCG CCGACGTCAG TCCCGATGCC CTCGATCCGG CGCTGGTACG TGGCTGTGAC
14581 AGCGTGCTCT GGACCGTGCA GGCCCTGGCC GGCATGGGCT TTCGAGACGC CCCGCGATTG
14641 TGGCTTCTGA CCCGCGGCGC ACAGGCCGTC GGCGCCGGCG ACGTCTCCGT GACACAGGCA
14701 CCGCTGCTGG GGCTGGGCCG CGTCATCGCC ATGGAGCACG CGGATCTGCG CTGCGCTCGG
14761 GTCGACCTCG ATCCGACCCG GCCCGATGGG GAGCTCGGTG CCCTGCTGGC CGAGCTGCTG
14821 GCCGACGACG CCGAAGCGGA AGTCGCGTTG CGCGGTGGCG AGCGATGCGT CGCTCGGATC
14881 GTCCGCCGGC AGCCCGAGAC CCGGCCCCGG GGGAGGATCG AGAGCTGCGT TCCGACCGAC
14941 GTCACCATCC GCGCGGACAG CACCTACCTT GTGACCGGCG GTCTGGGTGG GCTCGGTCTG
15001 AGCGTGGCCG GATGGCTGGC CGAGCGCGGC GCTGGTCACC TGGTGCTGGT GGGCCGCTCC
15061 GGCGCGGCGA GCGTGGAGCA ACGGGCAGCC GTCGCGGCGC TCGAGGCCCG CGGCGCGCGC
15121 GTCACCGTGG CGAAGGCAGA TGTCGCCGAT CGGGCGCAGC TCGAGCGGAT CCTCCGCGAG
15181 GTTACCACGT CGGGGATGCC GCTGCGGGGC GTCGTCCATG CGGCCGGCAT CTTGGACGAC
15241 GGGCTGCTGA TGCAGCAGAC TCCCGCGCGG TTTCGTAAGG TGATGGCGCC CAAGGTCCAG
15301 GGGGCCTTGC ACCTGCACGC GTTGACGCGC GAAGCGCCGC TTTCCTTCTT CGTGCTGTAC
15361 GCTTCGGGAG TAGGGCTCTT GGGCTCGCCG GGCCAGGGCA ACTACGCCGC GGCCAACACG
15421 TTCCTCGACG CTCTGGCGCA CCACCGGAGG GCGCAGGGGC TGCCAGCGTT GAGCGTCGAC
15481 TGGGGCCTGT TCGCGGAGGT GGGCATGGCG GCCGCGCAGG AAGATCGCGG CGCGCGGCTG
15541 GTCTCCCGCG GAATGCGGAG CCTCACCCCC GACGAGGGGC TGTCCGCTCT GGCACGGCTG
15601 CTCGAAAGCG GCCGCGTGCA GGTGGGGGTG ATGCCGGTGA ACCCGCGGCT GTGGGTGGAG
15661 CTCTACCCCG CGGCGGCGTC TTCGCGAATG TTGTCGCGCC TGGTGACGGC GCATCGCGCG
15721 AGCGCCGGCG GGCCAGCCGG GGACGGGGAC CTGCTCCGCC GCCTCGCTGC TGCCGAGCCG
15781 AGCGCGCGGA GCGGGCTCCT GGAGCCGCTC CTCCGCGCGC AGATCTCGCA GGTGCTGCGC
15841 CTCCCCGAGG GCAAGATCGA GGTGGACGCC CCGCTCACGA GCCTGGGCAT GAACTCGCTG
15901 ATGGGGCTCG AGCTGCGCAA CCGCATCGAG GCCATGCTGG GCATCACCGT ACCGGCAACG
15961 CTGTTGTGGA CCTATCCCAC GGTGGCGGCG CTGAGCGGGC ATCTGGCGCG GGAGGCATGC
16021 GAAGCCGCTC CTGTGGAGTC ACCGCACACC ACCGCCGATT CTGCTGTCGA GATCGAGGAG
16081 ATGTCGCAGG ACGATCTGAC GCAGTTGATC GCAGCAAAAT TCAAGGCGCT TACATGACTA
16141 CTCGCGGTCC TACGGCACAG CAGAATCCGC TGAAACAAGC GGCCATCATC ATTCAGCGGC
16201 TGGAGGAGCG GCTCGCTGGG CTCGCACAGG CGGAGCTGGA ACGGACCGAG CCGATCGCCA
16261 TCGTCGGTAT CGGCTGCCGC TTCCCTGGCG GTGCGGACGC TCCGGAAGCG TTTTGGGAGC
16321 TGCTCGACGC GGAGCGCGAC GCGGTCCAGC CGCTCGACAG GCGCTGGGCG CTGGTAGGTG
16381 TCGCTCCCGT CGAGGCCGTG CCGCACTGGG CGGGGCTGCT CACCGAGCCG ATAGATTGCT
16441 TCGATGCTGC GTTCTTCGGC ATCTCGCCTC GGGAGGCGCG ATCGCTCGAC CCGCAGCATC
16501 GTCTGTTGCT GGAGGTCGCT TGGGAGGGGC TCGAGGACGC CGGTATCCCG CCCCGGTCCA
16561 TCGACGGGAG CCGCACCGGT GTGTTCGTCG GCGCTTTCAC GGCGGACTAC GCGCGCACGG
16621 TCGCTCGGTT GCCGCGCGAG GAGCGAGACG CGTACAGCGC CACCGGCAAC ATGCTCAGCA
16681 TCGCCGCCGG ACGGCTGTCG TACACGCTGG GGCTGCAGGG ACCTTGCCTG ACCGTCGACA
16741 CGGCGTGCTC GTCATCGCTG GTGGCGATTC ACCTCGCCTG CCGCAGCCTG CGCGCAGGAG
16801 AGAGCGATCT CGCGTTGGCG GGAGGGGTCA GCACGCTCCT CTCCCCCGAC ATGATGGAAG
16861 CCGCGGCGCG CACGCAAGCG CTGTCGCCCG ATGGTCGTTG CCGGACCTTC GATGCTTCGG
16921 CCAACGGGTT CGTCCGTGGC GAGGGCTGTG GCCTGGTCGT CCTCAAACGG CTCTCCGACG
16981 CGCAACGGGA TGGCGACCGC ATCTGGGCGC TGATCCGGGG CTCGGCCATC AACCATGATG
17041 GCCGGTCGAC CGGGTTGACC GCGCCCAACG TGCTGGCTCA GGAGACGGTC TTGCGCGAGG
17101 CGCTGCGGAG CGCCCACGTC GAAGCTGGGG CCGTCGATTA CGTCGAGACC CACGGAACAG
17161 GGACCTCGCT GGGCGATCCC ATCGAGGTCG AGGCGCTGCG GGCGACGGTG GGGCCGGCGC
17221 GCTCCGACGG CACACGCTGC GTGCTGGGCG CGGTGAAGAC CAACATCGGC CATCTCGAGG
17281 CCGCGGCAGG CGTAGCGGGC CTGATCAAGG CAGCGCTTTC GCTGACGCAC GAGCGCATCC
17341 CGAGAAACCT CAACTTCCGC ACGCTCAATC CGCGGATCCG GCTCGAGGGC AGCGCGCTCG
17401 CGTTGGCGAC CGAGCCGGTG CCGTGGCCGC GCACGGACCG TCCGCGCTTC GCGGGGGTGA
17461 GCTCGTTCGG GATGAGCGGA ACGAACGCGC ATGTGGTGCT GGAAGAGGCG CCGGCGGTGG
17521 AGCTGTGGCC TGCCGCGCCG GAGCGCTCGG CGGAGCTTTT GGTGCTGTCG GGCAAGAGCG
17581 AGGGGGCGCT CGACGCGCAG GCGGCGCGGC TGCGCGAGCA CCTGGACATG CACCCGGAGC
17641 TCGGGCTCGG GGACGTGGCG TTCAGCCTGG CGACGACGCG CAGCGCGATG ACCCACCGGC
17701 TCGCGGTGGC GGTGACGTCG CGCGAGGGGC TGCTGGCGGC GCTTTCGGCC GTGGCGCAGG

17761 GGCAGACGCC GGCGGGGGCG GCGCGCTGCA TCGCGAGCTC CTCGCGCGGC AAGCTGGCGT
17821 TGCTGTTCAC CGGACAGGGC GCGCAGACGC CGGGCATGGG CCGGGGGCTC TGCGCGGCGT
17881 GGCCAGCGTT CCGGGAGGCG TTCGACCGGT GCGTGACGCT GTTCGACCGG GAGCTGGACC
17941 GCCCGCTGCG CGAGGTGATG TGGGCGGAGG CGGGGAGCGC CGAGTCGTTG TTGCTGGACC
18001 AGACGGCGTT CACCCAGCCC GCGCTCTTCG CGGTGGAGTA CGCGCTGACG GCGCTGTGGC
18061 GGTCGTGGGG CGTAGAGCCG GAGCTCCTGG TTGGGCATAG CATCGGGGAG CTGGTGGCGG
18121 CGTGCGTGGC GGGGGTGTTC TCGCTGGAAG ATGGGGTGAG GCTCGTGGCG GCGCGCGGGC
18181 GGCTGATGCA GGGGCTCTCG GCGGGCGGCG CGATGGTGTC GCTCGGAGCG CCGGAGGCGG
18241 AGGTGGCCGC GGCGGTGGCG CCGCACGCGG CGTGGGTGTC GATCGCGGCG GTCAATGGGC
18301 CGGAGCAGGT GGTGATCGCG GGCGTGGAGC AAGCGGTGCA GGCGATCGCG GCGGGGTTCG
18361 CGGCGCGCGG CGTGCGCACC AAGCGGCTGC ATGTCTCGCA CGCGTTCCAC TCGCCGCTGA
18421 TGGAACCGAT GCTGGAGGAG TTCGGGCGGG TGGCGGCGTC GGTGACGTAC CGGCGGCCAA
18481 GCGTTTCGCT GGTGAGCAAC CTGAGCGGGA AGGTGGTCAC GGACGAGCTG AGCGCGCCGG
18541 GCTACTGGGT GCGGCACGTG CGGGAGGCGG TGCGCTTCGC GGACGGGGTG AAGGCGCTGC
18601 ACGAAGCCGG CGCGGGCACG TTCCTCGAAG TGGGCCCGAA GCCGACGCTG CTCGGCCTGT
18661 TGCCAGCTTG CCTGCCGGAG GCGGAGCCGA CGTTGCTGGC GTCGTTGCGC GCCGGGCGCG
18721 AGGAGGCTGC GGGGGTGCTC GAGGCGCTGG GCAGGCTGTG GGCCGCTGGC GGCTCGGTCA
18781 GCTGGCCGGG CGTCTTCCCC ACGGCTGGGC GGCGGGTGCC GCTGCCGACC TATCCGTGGC
18841 AGCGGCAGCG GTACTGGATC GAGGCGCCGG CCGAAGGGCT CGGAGCCACG GCCGCCGATG
18901 CGCTGGCGCA GTGGTTCTAC CGGGTGGACT GGCCCGAGAT GCCTCGCTCA TCCGTGGATT
18961 CGCGGCGAGC CCGGTCCGGC GGGTGGCTGG TGCTGGCCGA CCGGGGTGGA GTCGGGGAGG
19021 CGGCCGCGGC GGCGCTTTCG TCGCAGGGAT GTTCGTGCGC CGTGCTCCAT GCGCCCGCCG
19081 AGGCCTCCGC GGTCGCCGAG CAGGTGACCC AGGCCCTCGG TGGCCGCAAC GACTGGCAGG
19141 GGGTGCTGTA CCTGTGGGGT CTGGACGCCG TCGTGGAGGC GGGGGCATCG GCCGAAGAGG
19201 TCGGCAAAGT CACCCATCTT GCCACGGCGC CGGTGCTCGC GCTGATTCAG GCGGTGGGCA
19261 CGGGGCCGCG CTCACCCCGG CTCTGGATCG TGACCCGAGG GGCCTGCACG GTGGGCGGCG
19321 AGCCTGACGC TGCCCCCTGT CAGGCGGCGC TGTGGGGTAT GGGCCGGGTC GCGGCGCTGG
19381 AGCATCCCGG CTCCTGGGGC GGGCTCGTGG ACCTGGATCC GGAGGAGAGC CCGACGGAGG
19441 TCGAGGCCCT GGTGGCCGAG CTGCTTTCGC CGGACGCCGA GGATCAGCTG GCATTCCGCC
19501 AGGGGCGCCG GCGCGCAGCG CGGCTCGTGG CCGCCCCACC GGAGGGAAAC GCAGCGCCGG
19561 TGTCGCTGTC TGCGGAGGGG AGTTACTTGG TGACGGGTGG GCTGGGCGCC CTTGGCCTCC
19621 TCGTTGCGCG GTGGTTGGTG GAGCGCGGGG CGGGGCACCT TGTGCTGATC AGCCGGCACG
19681 GATTGCCCGA CCGCGAGGAA TGGGGCCGAG ATCAGCCGCC AGAGGTGCGC GCGCGCATTG
19741 CGGCGATCGA GGCGCTGGAG GCGCAGGGCG CGCGGGTCAC CGTGGCGGCG GTCGACGTGG
19801 CCGATGCCGA AGGCATGGCG GCGCTCTTGG CGGCCGTCGA GCCGCCGCTG CGGGGGGTCG
19861 TGCACGCCGC GGGTCTGCTC GACGACGGGC TGCTGGCCCA CCAGGACGCC GGTCGGCTCG
19921 CCCGGGTGTT GCGCCCCAAG GTGGAGGGGG CATGGGTGCT GCACACCCTT ACCCGCGAGC
19981 AGCCGCTGGA CCTCTTCGTA CTGTTTTCCT CGGCGTCGGG CGTCTTCGGC TCGATCGGCC
20041 AGGGCAGCTA CGCGGCAGGC AATGCCTTTT TGGACGCGCT GGCGGACCTC CGTCGAACGC
20101 AGGGGCTCGC CGCCCTGAGC ATCGCCTGGG GCCTGTGGGC GGAGGGGGGG ATGGGCTCGC
20161 AGGCGCAGCG CCGGGAACAT GAGGCATCGG GAATCTGGGC GATGCCGACG AGTCGTGCCC
20221 TGGCGGCGAT GGAATGGCTG CTCGGTACGC GCGCGACGCA GCGCGTGGTC ATCCAGATGG
20281 ATTGGGCCCA TGCGGGAGCG GCTCCGCGCG ACGCGAGCCG AGGCCGCTTC TGGGATCGGC
20341 TGGTAACTGT CACGAAAGCG GCCTCCTCCT CGGCCGTGCC AGCTGTAGAG CGCTGGCGCA
20401 ACGCGTCTGT TGTGGAGACC CGCTCGGCGC TCTACGAGCT TGTGCGCGGC GTGGTCGCCG
20461 GGGTGATGGG CTTTACCGAC CAAGGCACGC TCGACGTGCG ACGAGGCTTC GCCGAGCAGG
20521 GCCTCGACTC CCTGATGGCT GTGGAGATCC GCAAACGGCT TCAGGGTGAG CTGGGTATGC
20581 CGCTGTCGGC GACGCTGGCG TTCGACCATC CGACCGTGGA GCGGCTGGTG GAATACTTGC
20641 TGAGCCAGGC GCTGGAGCTG CAGGACCGCA CCGACGTGCG AAGCGTTCGG TTGCCGGCGA
20701 CAGAGGACCC GATCGCCATC GTGGGTGCCG CCTGCCGCTT CCCGGGCGGG GTCGAGGACC
20761 TGGAGTCCTA CTGGCAGCTG TTGACCGAGG GCGTGGTGGT CAGCACCGAG GTGCCGGCCG
20821 ACCGGTGGAA TGGGGCAGAC GGGCGCGGCC CCGGCTCGGG AGAGGCTCCG AGACAGACCT
20881 ACGTGCCCAG GGGTGGCTTT CTGCGCGAGG TGGAGACGTT CGATGCGGCG TTCTTCCACA
20941 TCTCGCCTCG GGAGGCGATG AGCCTGGACC CGCAACAGCG GCTGCTGCTG GAAGTGAGCT

```
21001 GGGAGGCGAT CGAGCGCGCG GGCCAGGACC CGTCGGCGCT GCGCGAGAGC CCCACGGGCG
21061 TGTTCGTGGG CGCGGGCCCC AACGAATATG CCGAGCGGGT GCAGGACCTC GCCGATGAGG
21121 CGGCGGGGCT CTACAGCGGC ACCGGCAACA TGCTCAGCGT TGCGGCGGGA CGGCTGTCAT
21181 TTTTCCTGGG CCTGCACGGG CCGACCCTGG CTGTGGATAC GGCGTGCTCC TCGTCGCTCG
21241 TGGCGCTGCA CCTCGGCTGC CAGAGCTTGC GACGGGGCGA GTGCGACCAA GCCCTGGTTG
21301 GCGGGGTCAA CATGCTGCTC TCGCCGAAGA CCTTCGCGCT GCTCTCACGG ATGCACGCGC
21361 TTTCGCCCGG CGGGCGGTGC AAGACGTTCT CGGCCGACGC GGACGGCTAC GCGCGGGCCG
21421 AGGGCTGCGC CGTGGTGGTG CTCAAGCGGC TCTCCGACGC GCAGCGCGAC CGCGACCCCA
21481 TCCTGGCGGT GATCCGGGGT ACGGCGATCA ATCATGATGG CCCGAGCAGC GGGCTGACAG
21541 TGCCCAGCGG CCCTGCCCAG GAGGCGCTGT TACGCCAGGC GCTGGCGCAC GCAGGGGTGG
21601 TTCCGGCCGA CGTCGATTTC GTGGAATGCC ACGGGACCGG GACGGCGCTG GGCGACCCGA
21661 TCGAGGTGCG GGCGCTGAGC GACGTGTACG GGCAAGCCCG CCCTGCGGAC CGACCGCTGA
21721 TCCTGGGAGC CGCCAAGGCC AACCTTGGGC ACATGGAGCC CGCGGCGGGC CTGGCCGGCT
21781 TGCTCAAGGC GGTGCTCGCG CTGGGGCAAG AGCAAATACC AGCCCAGCCG GAGCTGGGCG
21841 AGCTCAACCC GCTCTTGCCG TGGGAGGCGC TGCCGGTGGC GGTGGCCCGC GCAGCGGTGC
21901 CGTGGCCGCG CACGGACCGT CCGCGCTTCG CGGGGGTGAG CTCGTTCGGG ATGAGCGGAA
21961 CGAACGCGCA TGTGGTGCTG GAAGAGGCGC CGGCGGTGGA GCTGTGGCCT GCCGCGCCGG
22021 AGCGCTCGGC GGAGCTTTTG GTGCTGTCGG GCAAGAGCGA GGGGGCGCTC GACGCGCAGG
22081 CGGCGCGGCT GCGCGAGCAC CTGGACATGC ACCCGGAGCT CGGGCTCGGG GACGTGGCGT
22141 TCAGCCTGGC GACGACGCGC AGCGCGATGA ACCACCGGCT CGCGGTGGCG GTGACGTCGC
22201 GCGAGGGGCT GCTGGCGGCG CTTTCGGCCG TGGCGCAGGG GCAGACGCCG CCGGGGGCGG
22261 CGCGCTGCAT CGCGAGCTCG TCGCGCGGCA AGCTGGCGTT CCTGTTCACC GGACAGGGCG
22321 CGCAGACGCC GGGCATGGGC CGGGGGCTTT GCGCGGCGTG GCCAGCGTTC CGAGAGGCGT
22381 TCGACCGGTG CGTGGCGCTG TTCGACCGGG AGCTGGACCG CCCGCTGTGC GAGGTGATGT
22441 GGGCGGAGCC GGGGAGCGCC GAGTCGTTGT TGCTCGACCA GACGGCGTTC ACCCAGCCCG
22501 CGCTCTTCAC GGTGGAGTAC GCGCTGACGG CGCTGTGGCG GTCGTGGGGC GTAGAGCCGG
22561 AGCTGGTGGC TGGGCATAGC GCCGGGGAGC TGGTGGCGGC GTGCGTGGCG GGGGTGTTCT
22621 CGCTGGAAGA TGGGGTGAGG CTCGTGGCGG CGCGCGGGCG GCTGATGCAG GGGCTCTCGG
22681 CGGGCGGCGC GATGGTGTCG CTCGGAGCGC CGGAGGCGGA GGTGGCCGCG GCGGTGGCGC
22741 CGCACGCGGC GTGGGTGTCG ATCGCGGCGG TCAATGGGCC GGAGCAGGTG GTGATCGCGG
22801 GCGTGGAGCA AGCGGTGCAG GCGATCGCGG CGGGGTTCGC GGCGCGCGGC GTGCGCACCA
22861 AGCGGCTGCA TGTCTCGCAC GCATCCCACT CGCCGCTGAT GGAACCGATG CTGGAGGAGT
22921 TCGGGCGGGT GGCGGCGTCG GTGACGTACC GGCGGCCAAG CGTTTCGCTG GTGAGCAACC
22981 TGAGCGGGAA GGTGGTCACG GACGAGCTGA GCGCGCCGGG CTACTGGGTG CGGCACGTGC
23041 GGGAGGCGGT GCGCTTCGCG GACGGGGTGA AGGCGCTGCA CGAAGCCGGC GCGGGGACGT
23101 TCCTCGAAGT GGGCCCGAAG CCGACGCTGC TCGGCCTGTT GCCAGCTTGC CTGCCGGAGG
23161 CGGAGCCGAC GCTGCTGGCG TCGTTGCGCG CCGGGCGCGA GGAGGCTGCG GGGGTGCTCG
23221 AGGCGCTGGG CAGGCTGTGG GCCGCCGGCG GCTCGGTCAG CTGGCCGGGC GTCTTCCCCA
23281 CGGCTGGGCG GCGGGTGCCG CTGCCGACCT ATCCGTGGCA GCGGCAGCGG TACTGGCCCG
23341 ACATCGAGCC TGACAGCCGT CGCCACGCAG CCGCGGATCC GACCCAAGGC TGGTTCTATC
23401 GCGTGGACTG GCCGGAGATA CCTCGCAGCC TCCAGAAATC AGAGGAGGCG AGCCGCGGGA
23461 GCTGGCTGGT ATTGGCGGAT AAGGGTGGAG TCGGCGAGGC GGTCGCTGCA GCGCTGTCGA
23521 CACGTGGACT TCCATGCGTC GTGCTCCATG CGCCGGCAGA GACATCCGCG ACCGCCGAGC
23581 TGGTGACCGA GGCTGCCGGC GGTCGAAGCG ATTGGCAGGT AGTGCTCTAC CTGTGGGGTC
23641 TGGACGCCGT CGTCGGCGCG GAGGCGTCGA TCGATGAGAT CGGCGACGCG ACCCGTCGTG
23701 CTACCGCGCC GGTGCTCGGC TTGGCTCGGT TTCTGAGCAC CGTGTCTTGT TCGCCCCGAC
23761 TCTGGGTCGT GACCCGGGGG GCATGCATCG TTGGCGACGA GCCTGCGATC GCCCCTTGTC
23821 AGGCGGCGTT ATGGGGCATG GGCCGGGTGG CGGCGCTCGA GCATCCCGGG GCCTGGGGCG
23881 GGCTCGTGGA CCTGGATCCC CGAGCGAGCC CGCCCCAAGC CAGCCCGATC GACGGCGAGA
23941 TGCTCGTCAC CGAGCTATTG TCGCAGGAGA CCGAGGACCA GCTCGCCTTC CGCCATGGGC
24001 GCCGGCACGC GGCACGGCTG GTGGCCGCCC CGCCACGGGG GGAAGCGGCA CCGGCGTCGC
24061 TGTCTGCGGA GGCGAGCTAC CTGGTGACGG GAGGCCTCGG TGGGCTGGGC CTGATCGTGG
24121 CCCAGTGGCT GGTGGAGCTG GGAGCGCGGC ACTTGGTGCT GACCAGCCGG CGCGGGTTGC
24181 CCGACCGGCA GGCGTGGCGC GAGCAGCAGC CGCCTGAGAT CCGCGCGCGG ATCGCAGCGG
```

24241 TCGAGGCGCT GGAGGCGCGG GGTGCACGGG TGACCGTGGC AGCGGTGGAC GTGGCCGACG
24301 TCGAACCGAT GACAGCGCTG GTTTCGTCGG TCGAGCCCCC GCTGCGAGGG GTGGTGCACG
24361 CCGCTGGCGT CAGCGTCATG CGTCCACTGG CGGAGACGGA CGAGACCCTG CTCGAGTCGG
24421 TGCTCCGTCC CAAGGTGGCC GGGAGCTGGC TGCTGCACCG GCTGCTGCAC GGCCGGCCTC
24481 TCGACCTGTT CGTGCTGTTC TCGTCGGGCG CAGCGGTGTG GGGTAGCCAT AGCCAGGGTG
24541 CGTACGCGGC GGCCAACGCT TTCCTCGACG GGCTCGCGCA TCTTCGGCGT TCGCAATCGC
24601 TGCCTGCGTT GAGCGTCGCG TGGGGTCTGT GGGCCGAGGG AGGCATGGCG GACGCGGAGG
24661 CTCATGCACG TCTGAGCGAC ATCGGGGTTC TGCCCATGTC GACGTCGGCA GCGTTGTCGG
24721 CGCTCCAGCG CCTGGTGGAG ACCGGCGCGG CTCAGCGCAC GGTGACCCGG ATGGACTGGG
24781 CGCGCTTCGC GCCGGTGTAC ACCGCTCGAG GGCGTCGCAA CCTGCTTTCG GCGCTGGTCG
24841 CAGGGCGCGA CATCATCGCG CCTTCCCCTC CGGCGGCAGC AACCCGGAAC TGGCGTGGCC
24901 TGTCCGTTGC GGAAGCCCGC ATGGCTCTGC ACGAGGTCGT CCATGGGGCC GTCGCTCGGG
24961 TGCTGGGCTT CCTCGACCCG AGCGCGCTCG ATCCTGGGAT GGGGTTCAAT GAGCAGGGCC
25021 TCGACTCGTT GATGGCGGTG GAGATCCGCA ACCTCCTTCA GGCTGAGCTG GACGTGCGGC
25081 TTTCGACGAC GCTGGCCTTT GATCATCCGA CGGTACAGCG GCTGGTGGAG CATCTGCTCG
25141 TCGATGTACT GAAGCTGGAG GATCGCAGCG ACACCCAGCA TGTTCGGTCG TTGGCGTCAG
25201 ACGAGCCCAT CGCCATCGTG GGAGCCGCCT GCCGCTTCCC GGGCGGGGTG GAGGACCTGG
25261 AGTCCTACTG GCAGCTGTTG GCCGAGGGCG TGGTGGTCAG CGCCGAGGTG CCGGCCGACC
25321 GGTGGGATGC GGCGGACTGG TACGACCCTG ATCCGGAGAT CCCAGGCCGG ACTTACGTGA
25381 CCAAAGGCGC CTTCCTGCGC GATTTGCAGA GATTGGATGC GACCTTCTTC CGCATCTCGC
25441 CTCGCGAGGC GATGAGCCTC GACCCGCAGC AGCGGTTGCT CCTGGAGGTA AGCTGGGAGG
25501 CGCTCGAGAG CGCGGGTATC GCTCCGGATA CGCTGCGAGA TAGCCCCACC GGGGTGTTCG
25561 TGGGTGCGGG GCCCAATGAG TACTACACGC AGCGGCTGCG AGGCTTCACC GACGGAGCGG
25621 CAGGGCTGTA CGGCGGCACC GGGAACATGC TCAGCGTTGC GGCTGGACGG CTGTCGTTTT
25681 TCCTGGGTCT GCACGGCCCG ACGCTGGCCA TGGATACGGC GTGCTCGTCC TCCCTGGTCG
25741 CGCTGCACCT CGCCTGCCAG AGCCTGCGAC TGGGCGAGTG CGATCAAGCG CTGGTTGGCG
25801 GGGTCAACGT GCTGCTCGCG CCGGAGACCT TCGTGCTGCT CTCACGGATG CGCGCGCTTT
25861 CGCCCGACGG GCGGTGCAAG ACGTTCTCGG CCGACGCGGA CGGCTACGCG CGGGGCGAGG
25921 GGTGCGCCGT GGTGGTGCTC AAGCGGCTGC GCGATGCGCA GCGCGCCGGC GACTCCATCC
25981 TGGCGCTGAT CCGGGGAAGC GCGGTGAACC ACGACGGCCC GAGCAGCGGG CTGACCGTGC
26041 CCAACGGACC CGCCCAGCAA GCATTGCTGC GCCAGGCGCT TTCGCAAGCA GGCGTGTCTC
26101 CGGTCGACGT TGATTTTGTG GAGTGTCACG GGACAGGGAC GGCGCTGGGC GACCCGATCG
26161 AGGTGCAGGC GCTGAGCGAG GTGTATGGTC CAGGGCGCTC CGAGGATCGA CCGCTGGTGC
26221 TGGGGGCCGT CAAGGCCAAC GTCGCGCATC TGGAGGCGGC ATCCGGCTTG GCCAGCCTGC
26281 TCAAGGCCGT GCTTGCGCTG CGGCACGAGC AGATCCCGGC CCAGCCGGAG CTGGGGGAGC
26341 TCAACCCGCA CTTGCCGTGG AACACGCTGC CGGTGGCGGT GCCACGTAAG GCGGTGCCGT
26401 GGGGGCGCGG CGCACGGCCG CGTCGGGCCG GCGTGAGCGC GTTCGGGTTG AGCGGAACCA
26461 ACGTGCATGT CGTGCTGGAG GAGGCACCGG AGGTGGAGCT GGTGCCCGCG GCGCCGGCGC
26521 GACCGGTGGA GCTGGTTGTG CTATCGGCCA AGAGCGCGGC GGCGCTGGAC GCCGCGGCGG
26581 AACGGCTCTC GGCGCACCTG TCCGCGCACC CGGAGCTGAG CCTCGGCGAC GTGGCGTTCA
26641 GCCTGGCGAC GACGCGCAGC CCGATGGAGC ACCGGCTCGC CATCGCGACG ACCTCGCGCG
26701 AGGCCCTGCG AGGCGCGCTG GACGCCGCGG CGCAGCGGCA GACGCCGCAG GGCGCGGTGC
26761 GCGGCAAGGC CGTGTCCTCA CGCGGTAAGT TGGCTTTCCT GTTCACCGGA CAGGGCGCGC
26821 AAATGCCGGG CATGGGCCGT GGGCTGTACG AGGCGTGGCC AGCGTTCCGG GAGGCGTTCG
26881 ACCGGTGCGT GGCGCTCTTC GATCGGGAGC TCGACCAGCC TCTGCGCGAG GTGATGTGGG
26941 CTGCGCCGGG CCTCGCTCAG GCGGCGCGGC TCGATCAGAC CGCGTACGCG CAGCCGGCTC
27001 TCTTTGCGCT GGAGTACGCG CTGGCTGCCC TGTGGCGTTC GTGGGGCGTG GAGCCGCACG
27061 TACTCCTCGG TCATAGCATC GGCGAGCTGG TCGCCGCCTG CGTGGCGGGC GTGTTCTCGC
27121 TCGAAGACGC GGTGAGGTTG GTGGCCGCGC GCGGGCGGCT GATGCAGGCG CTGCCCGCCG
27181 GCGGTGCCAT GGTCGCCATC GCAGCGTCCG AGGCCGAGGT GGCCGCCTCC GTGGCACCCC
27241 ACGCCGCCAC GGTGTCGATC GCCGCGGTCA ACGGTCCTGA CGCCGTCGTG ATCGCTGGCG
27301 CCGAGGTACA GGTGCTCGCC CTCGGCGCGA CGTTCGCGGC GCGTGGGATA CGCACGAAGA
27361 GGCTCGCCGT CTCCCATGCG TTCCACTCGC CGCTCATGGA TCCGATGCTG GAAGACTTCC
27421 AGCGGGTCGC TGCGACGATC GCGTACCGCG CGCCAGACCG CCCGGTGGTG TCGAATGTCA

```
27481 CCGGCCACGT CGCAGGCCCC GAGATCGCCA CGCCCGAGTA TTGGGTCCGG CATGTGCGAA
27541 GCGCCGTGCG CTTCGGCGAT GGGGCAAAGG CGTTGCATGC CGCGGGTGCC GCCACGTTCG
27601 TCGAGATTGG CCCGAAGCCG GTCCTGCTCG GGCTATTGCC AGCGTGCCTC GGGGAAGCGG
27661 ACGCGGTCCT CGTGCCGTCG CTACGCGCGG ACCGCTCGGA ATGCGAGGTG GTCCTCGCGG
27721 CGCTCGGGAC TTGGTATGCC TGGGGGGGTG CGCTCGACTG GAAGGGCGTG TTCCCCGATG
27781 GCGCGCGCCG CGTGGCTCTG CCCATGTATC CATGGCAGCG TGAGCGCCAT TGGATGGACC
27841 TCACCCCGCG AAGCGCCGCG CCTGCAGGGA TCGCAGGTCG CTGGCCGCTG GCTGGTGTCG
27901 GGCTCTGCAT GCCCGGCGCT GTGTTGCACC ACGTGCTCTC GATCGGACCA CGCCATCAGC
27961 CCTTCCTCGG TGATCACCTC GTGTTTGGCA AGGTGGTGGT GCCCGGCGCC TTTCATGTCG
28021 CGGTGATCCT CAGCATCGCC GCCGAGCGCT GGCCCGAGCG GGCGATCGAG CTGACAGGCG
28081 TGGAGTTCCT GAAGGCGATC GCGATGGAGC CCGACCAGGA GGTCGAGCTC CACGCCGTGC
28141 TCACCCCCGA AGCCGCCGGG GATGGCTACC TGTTCGAGCT GGCGACCCTG GCGGCGCCGG
28201 AGACCGAACG CCGATGGACG ACCCACGCCC GCGGTCGGGT GCAGCCGACA GACGGCGCGC
28261 CCGGCGCGTT GCCGCGCCTC GAGGTGCTGG AGGACCGCGC GATCCAGCCC CTCGACTTCG
28321 CCGGATTCCT CGACAGGTTA TCGGCGGTGC GGATCGGCTG GGGTCCGCTT TGGCGATGGC
28381 TGCAGGACGG GCGCGTCGGC GACGAGGCCT CGCTTGCCAC CCTCGTGCCG ACCTATCCGA
28441 ACGCCCACGA CGTGGCGCCC TTGCACCCGA TCCTGCTGGA CAACGGCTTT GCGGTGAGCC
28501 TGCTGGCAAC CCGGAGCGAG CCGGAGGACG ACGGGACGCC CCCGCTGCCG TTCGCCGTGG
28561 AACGGGTGCG GTGGTGGCGG GCGCCGGTTG GAAGGGTGCG GTGTGGCGGC GTGCCGCGGT
28621 CGCAGGCATT CGGTGTCTCG AGCTTCGTGC TGGTCGACGA AACTGGCGAG GTGGTCGCTG
28681 AGGTGGAGGG ATTTGTTTGC CGCCGGGCGC CGCGAGAGGT GTTCCTGCGG CAGGAGTCGG
28741 GCGCGTCGAC TGCAGCCTTG TACCGCCTCG ACTGGCCCGA AGCCCCCTTG CCCGATGCGC
28801 CTGCGGAACG GATGGAGGAG AGCTGGGTCG TGGTGGCAGC ACCTGGCTCG GAGATGGCCG
28861 CGGCGCTCGC AACACGGCTC AACCGCTGCG TACTCGCCGA ACCCAAAGGC CTCGAGGCGG
28921 CCCTCGCGGG GGTGTCTCCC GCAGGTGTGA TCTGCCTCTG GGAACCTGGA GCCCACGAGG
28981 AAGCTCCGGC GGCGGCGCAG CGTGTGGCGA CCGAGGGCCT TTCGGTGGTG CAGGCGCTCA
29041 GGGATCGCGC GGTGCGCCTG TGGTGGGTGA CCACGGGCGC CGTGGCTGTC GAGGCCGGTG
29101 AGCGGGTGCA GGTCGCCACA GCGCCGGTAT GGGGCCTGGG CCGGACAGTG ATGCAGGAGC
29161 GCCCGGAGCT CAGCTGCACT CTGGTGGATT TGGAGCCGGA GGTCGATGCC GCGCGTTCAG
29221 CTGACGTTCT GCTGCGGGAG CTCGGTCGCG CTGACGACGA GACCCAGGTG GTTTTCCGTT
29281 CCGGAGAGCG CCGCGTAGCG CGGCTGGTCA AAGCGACAAC CCCCGAAGGG CTCTTGGTCC
29341 CTGACGCAGA ATCCTATCGA CTGGAGGCTG GGCAGAAGGG CACATTGGAC CAGCTCCGCC
29401 TCGCGCCGGC ACAGCGCCGG GCACCCGGCC CGGGCGAGGT CGAGATCAAG GTAACCGCCT
29461 CGGGGCTCAA CTTCCGGACC GTCCTCGCTG TGCTGGGAAT GTATCCGGGC GACGCTGGGC
29521 CGATGGGCGG AGATTGTGCC GGTATCGTCA CGGCGGTGGG CCAGGGGGTG CACCACCTCT
29581 CGGTCGGCGA TGCTGTCATG ACGCTGGGGA CGTTGCATCG ATTCGTCACG GTCGACGCGC
29641 GGCTGGTGGT CCGGCAGCCT GCAGGGCTGA CTCCCGCGCA GGCAGCTACG GTGCCGGTTG
29701 CGTTCCTGAC GGCCTGGCTC GCTCTGCACG ACCTGGGGAA TCTGCGGCGC GGCGAGCGGG
29761 TGCTGATCCA TGCTGCGGCC GGCGGCGTGG GCATGGCCGC GGTGCAAATC GCCCGATGGA
29821 TAGGGGCCGA GGTGTTCGCC ACGGCGAGCC CGTCCAAGTG GGCAGCGGTT CAGGCCATGG
29881 GCGTGCCGCG CACGCACATC GCCAGCTCGC GGACGCTGGA GTTTGCTGAG ACGTTCCGGC
29941 AGGTCACCGG CGGCCGGGGC GTGGACGTGG TGCTCAACGC GCTGGCCGGC GAGTTCGTGG
30001 ACGCGAGCCT GTCCCTGCTG ACGACGGGCG GGCGGTTCCT CGAGATGGGC AAGACCGACA
30061 TACGGGATCG AGCCGCGGTC GCGGCGGCGC ATCCCGGTGT TCGCTATCGG GTATTCGACA
30121 TCCTGGAGCT CGCTCCGGAT CGAACTCGAG AGATCCTCGA GCGCGTGGTC GAGGGCTTTG
30181 CTGCGGGACA TCTGCGCGCA TTGCCGGTGC ATGCGTTCGC GATCACCAAG GCCGAGGCAG
30241 CGTTTCGGTT CATGGCGCAA GCGCGGCATC AGGGCAAGGT CGTGCTGCTG CCGGCGCCCT
30301 CCGCAGCGCC CTTGGCGCCG ACGGGCACCG TACTGCTGAC CGGTGGGCTG GGAGCGTTGG
30361 GGCTCCACGT GGCCCGCTGG CTCGCCCAGC AGGGCGCGCC GCACATGGTG CTCACAGGTC
30421 GGCGGGGCCT GGATACGCCG GGCGCTGCCA AAGCCGTCGC GGAGATCGAA GCGCTCGGCG
30481 CTCGGGTGAC GATCGCGGCG TCGGATGTCG CCGATCGGAA CGCGCTGGAG GCTGTGCTCC
30541 AGGCCATTCC GGCGGAGTGG CCGTTACAGG GCGTGATCCA TGCAGCCGGA GCGCTCGATG
30601 ATGGTGTGCT TGATGAGCAG ACCACCGACC GCTTCTCGCG GGTGCTGGCA CCGAAGGTGA
30661 CTGGCGCCTG GAATCTGCAT GAGCTCACGG CGGGCAACGA TCTCGCTTTC TTCGTGCTGT
```

30721 TCTCCTCCAT GTCGGGGCTC TTGGGCTCGG CCGGGCAGTC CAACTATGCG GCGGCCAACA
30781 CCTTCCTCGA CGCGCTGGCC GCGCATCGGC GGGCCGAAGG CCTGGCGGCG CAGAGCCTCG
30841 CGTGGGGCCC ATGGTCGGAC GGAGGCATGG CAGCGGGGCT CAGCGCGGCG CTGCAGGCGC
30901 GGCTCGCTCG GCATGGGATG GGAGCGCTGT CGCCCGCTCA GGGCACCGCG CTGCTCGGGC
30961 AGGCGCTGGC TCGGCCGGAA ACGCAGCTCG GGGCGATGTC GCTCGACGTG CGTGCGGCAA
31021 GCCAAGCTTC GGGAGCGGCA GTGCCGCCTG TGTGGCGCGC GCTGGTGCGC GCGGAGGCGC
31081 GCCATGCGGC GGCTGGGGCG CAGGGGGCAT TGGCCGCGCG CCTTGGGGCG CTGCCCGAGG
31141 CGCGTCGCGC CGACGAGGTG CGCAAGGTCG TGCAGGCCGA GATCGCGCGC GTGCTTTCAT
31201 GGGGCGCCGC GAGCGCCGTG CCCGTCGATC GGCCGCTGTC GGACTTGGGC CTCGACTCGC
31261 TCACGGCGGT GGAGCTGCGC AACGTGCTCG GCCAGCGGGT GGGTGCGACG CTGCCGGCGA
31321 CGCTGGCATT CGATCACCCG ACGGTCGACG CGCTCACGCG CTGGCTGCTC GATAAGGTCC
31381 TGGCCGTGGC CGAGCCGAGC GTATCGCCCG CAAAGTCGTC GCCGCAGGTC GCCCTCGACG
31441 AGCCCATTGC GGTGATCGGC ATCGGCTGCC GTTTCCCAGG CGGCGTGACC GATCCGGAGT
31501 CGTTTTGGCG GCTGCTCGAA GAGGGCAGCG ATGCCGTCGT CGAGGTGCCG CATGAGCGAT
31561 GGGACATCGA CGCGTTCTAT GATCCGGATC CGGATGTGCG CGGCAAGATG ACGACACGCT
31621 TTGGCGGCTT CCTGTCCGAT ATCGACCGGT TCGAGCCGGC CTTCTTCGGC ATCTCGCCGC
31681 GCGAAGCGAC GACCATGGAT CCGCAGCAGC GGCTGCTCCT GGAGACGAGC TGGGAGGCGT
31741 TCGAGCGCGC CGGGATTTTG CCCGAGCGGC TGATGGGCAG CGATACCGGC GTGTTCGTGG
31801 GGCTCTTCTA CCAGGAGTAC GCTGCGCTCG CCGGCGGCAT CGAGGCGTTC GATGGCTATC
31861 TAGGCACCGG CACCACGGCC AGCGTCGCCT CGGGCAGGAT CTCTTATGTG CTCGGGCTAA
31921 AGGGGCCGAG CCTGACGGTG GACACCGCGT GCTCCTCGTC GCTGGTCGCG GTGCACCTGG
31981 CCTGCCAGGC GCTGCGGCGG GGCGAGTGTT CGGTGGCGCT GGCCGGCGGC GTGGCGCTGA
32041 TGCTCACGCC GGCGACGTTC GTGGAGTTCA GCCGGCTGCG AGGCCTGGCT CCCGACGGAC
32101 GGTGCAAGAG CTTCTCGGCC GCAGCCGACG GCGTGGGGTG GAGCGAAGGC TGCGCCATGC
32161 TCCTGCTCAA ACCGCTTCGC GATGCTCAGC GCGATGGGGA TCCGATCCTG GCGGTGATCC
32221 GCGGCACCGC GGTGAACCAG GATGGGCGCA GCAACGGGCT GACGGCGCCC AACGGGTCGT
32281 CGCAGCAAGA GGTGATCCGT CGGGCCCTGG AGCAGGCGGG GCTGGCTCCG GCGGACGTCA
32341 GCTACGTCGA GTGCCACGGC ACCGGCACGA CGTTGGGCGA CCCCATCGAA GTGCAGGCCC
32401 TGGGCGCCGT GCTGGCACAG GGGCGACCCT CGGACCGGCC GCTCGTGATC GGGTCGGTGA
32461 AGTCCAATAT CGGACATACG CAGGCTGCGG CGGGCGTGGC CGGTGTCATC AAGGTGGCGC
32521 TGGCGCTCGA GCGCGGGCTT ATCCCGAGGA GCCTGCATTT CGACGCGCCC AATCCGCACA
32581 TTCCGTGGTC GGAGCTCGCC GTGCAGGTGG CCGCCAAACC CGTCGAATGG ACGAGAAACG
32641 GCGCGCCGCG ACGAGCCGGG GTGAGCTCGT TTGGCGTCAG CGGGACCAAC GCGCACGTGG
32701 TGCTGGAGGA GGCGCCAGCG GCGGCGTTCG CGCCCGCGGC GGCGCGTTCA GCGGAGCTTT
32761 TCGTGCTGTC GGCGAAGAGC GCCGCGGCGC TGGACGCGCA GGCGGCGCGG CTTTCGGCGC
32821 ATGTCGTTGC GCACCCGGAG CTCGGCCTCG GCGACCTGGC GTTCAGCCTG GCGACGACCC
32881 GCAGCCCGAT GACGTACCGG CTCGCGGTGG CGGCGACCTC GCGCGAGGCG CTGTCTGCGG
32941 CGCTCGACAC AGCGGCGCAG GGGCAGGCGC CGCCCGCAGC GGCTCGCGGC CACGCTTCCA
33001 CAGGCAGCGC CCCAAAGGTG GTTTTCGTCT TTCCTGGCCA GGGCTCCCAG TGGCTGGGCA
33061 TGGGCCAAAA GCTCCTCTCG GAGGAGCCCG TCTTCCGCGA CGCGCTCTCG GCGTGTGACC
33121 GAGCGATTCA GGCCGAAGCC GGCTGGTCGC TGCTCGCCGA GCTCGCGGCC GATGAGACCA
33181 CCTCGCAGCT CGGCCGCATC GACGTGGTGC AGCCGGCGCT GTTCGCGATC GAGGTCGCGC
33241 TGTCGGCGCT GTGGCGGTCG TGGGGCGTCG AGCCGGATGC AGTGGTAGGC CACAGCATGG
33301 GCGAAGTGGC GGCCGCGCAC GTCGCCGGCG CCCTGTCGCT CGAGGATGCT GTAGCGATCA
33361 TCTGCCGGCG CAGCCTGCTG CTGCGGCGGA TCAGCGGCCA AGGCGAGATG GCGGTCGTCG
33421 AGCTCTCCCT GGCCGAGGCC GAGGCAGCGC TCCTGGGCTA CGAAGATCGG CTCAGCGTGG
33481 CGGTGAGCAA CAGCCCGCGA TCGACGGTGC TGGCGGGCGA GCCGGCAGCG CTCGCAGAGG
33541 TGCTGGCGAT CCTTGCGGCA AAGGGGGTGT TCTGCCGTCG AGTCAAGGTG GACGTCGCCA
33601 GCCACAGCCC ACAGATCGAC CCGCTGCGCG ACGAGCTATT GGCAGCATTG GGCGAGCTCG
33661 AGCCGCGACA AGCGACCGTG TCGATGCGCT CGACGGTGAC GAGCACGATC GTGGCGGGCC
33721 CGGAGCTCGT GGCGAGCTAC TGGGCGGACA ACGTTCGACA GCCGGTGCGC TTCGCCGAAG
33781 CGGTGCAATC GTTGATGGAA GGCGGTCATG GGCTGTTCGT GGAGATGAGC CCGCATCCGA
33841 TCCTGACGAC GTCGGTCGAG GAGATCCGAC GGGCGACGAA GCGGGAGGGA GTCGCGGTGG
33901 GCTCGTTGCG GCGTGGACAG GACGAGCGCC TGTCCATGTT GGAGGCGCTG GGAGCGCTCT

```
33961 GGGTACACGG CCAGGCGGTG GGCTGGGAGC GGCTGTTCTC CGCGGGCGGC GCGGGCCTCC
34021 GTCGCGTGCC GCTGCCGACC TATCCCTGGC AGCGCGAGCG GTACTGGGTC GAAGCGCCGA
34081 CCGGCGGCGC GGCGAGCGGC AGCCGCTTTG CTCATGCGGG CAGTCACCCG CTCCTGGGTG
34141 AAATGCAGAC CCTGTCGACC CAGAGGAGCA CGCGCGTGTG GGAGACGACG CTGGATCTCA
34201 AACGGCTGCC GTGGCTCGGC GATCACCGGG TGCAGGGGGC GGTCGTGTTC CCGGGCGCGG
34261 CGTACCTGGA GATGGCGCTT TCGTCTGGGG CCGAGGCCTT GGGTGACGGT CCGCTCCAGG
34321 TCAGCGATGT GGTGCTCGCC GAGGCGCTGG CCTTCGCGGA TGATACGCCG GTGGCGGTGC
34381 AGGTCATGGC GACCGAGGAG CGACCAGGCC GCCTGCAATT CCACGTTGCG AGCCGGGTGC
34441 CGGGCCACGG CCGTGCTGCC TTTCGAAGCC ATGCCCGCGG GGTGCTGCGC CAGACCGAGC
34501 GCGCCGAGGT CCCGGCGAGG CTGGATCTGG CCGCGCTTCG TGCCCGGCTT CAGGCCAGCG
34561 CACCCGCTGC GGCTACCTAT GCGGCGCTGG CCGAGATGGG GCTCGAGTAC GGCCCAGCGT
34621 TCCAGGGGCT TGTCGAGCTG TGGCGGGGGG AGGGCGAGGC GCTGGGACGT GTGCGGCTCC
34681 CCGAGGCCGC CGGCTCCCCA GCCGCGTGCC GGCTCCACCC CGCGCTCTTG GATGCGTGCT
34741 TCCACGTGAG CAGCGCCTTC GCTGACCGCG GCGAGGCGAC GCCATGGGTA CCCGTCGAAA
34801 TCGGCTCGCT GCGGTGGTTC CAGCGGCCGT CGGGGGAGCT GTGGTGTCAT GCGCGGAGCG
34861 TGAGCCACGG AAAGCCAACA CCCGATCGGC GGAGTACCGA CTTTTGGGTG GTCGACAGCA
34921 CGGGCGCGAT CGTCGCCGAG ATCTCCGGGC TCGTGGCGCA GCGGCTCGCG GGAGGTGTAC
34981 GCCGGCGCGA AGAAGACGAC TGGTTCATGG AGCCGGCTTG GGAACCGACC GCGGTCCCCG
35041 GATCCGAGGT CACGGCGGGC CGGTGGCTGC TCATCGGCTC GGGCGGCGGG CTCGGCGCTG
35101 CGCTCTACTC GGCGCTGACG GAAGCTGGCC ATTCCGTCGT CCACGCGACA GGGCACGGCA
35161 CGAGCGCCGC CGGGTTGCAG GCACTCCTGA CGGCGTCCTT CGACGGCCAG GCCCCGACGT
35221 CGGTGGTGCA CCTCGGCAGC CTCGATGAGC GTGGCGTGCT CGACGCGGAT GCCCCCTTCG
35281 ACGCCGATGC CCTCGAGGAG TCGCTGGTGC GCGGCTGCGA CAGCGTGCTC TGGACCGTGC
35341 AGGCCGTGGC CGGGGCGGGC TTCCGAGATC CTCCGCGGTT GTGGCTCGTG ACACGCGGCG
35401 CTCAGGCCAT CGGCGCCGGC GACGTCTCCG TGGCGCAAGC GCCGCTCCTG GGGCTGGGCC
35461 GCGTTATCGC CTTGGAGCAC GCCGAGCTGC GCTGCGCTCG GATCGACCTC GATCCAGCGC
35521 GGCGCGACGG AGAGGTCGAT GAGCTGCTTG CCGAGCTGTT GGCCGACGAC GCCGAGGAGG
35581 AAGTCGCGTT TCGCGGCGGT GAGCGGCGCG TGGCCCGGCT CGTCCGAAGG CTGCCCGAGA
35641 CCGACTGCCG AGAGAAAATC GAGCCCGCGG AAGGCCGGCC GTTCCGGCTG GAGATCGATG
35701 GGTCCGGCGT GCTCGACGAC CTGGTGCTCC GAGCCACGGA GCGGCGCCCT CCTGGCCCGG
35761 GCGAGGTCGA GATCGCCGTC GAGGCGGCGG GGCTCAACTT TCTCGACGTG ATGAGGGCCA
35821 TGGGGATCTA CCCTGGGCCC GGGGACGGTC CGGTTGCGCT GGGCGCCGAG TGCTCCGGCC
35881 GAATTGTCGC GATGGGCGAA GGTGTCGAGA GCCTTCGTAT CGGCCAGGAC GTCGTGGCCG
35941 TCGCGCCCTT CAGTTTCGGC ACCCACGTCA CCATCGACGC CCGGATGGTC GCACCTCGCC
36001 CCGCGGCGCT GACGGCCGCG CAGGCAGCCG CGCTGCCCGT CGCATTCATG ACGGCCTGGT
36061 ACGGTCTCGT CCATCTGGGG AGGCTCCGGG CCGGCGAGCG CGTGCTCATC CACTCGGCGA
36121 CGGGGGGCAC CGGGCTCGCT GCTGTGCAGA TCGCCCGCCA CCTCGGCGCG GAGATATTTG
36181 CGACCGCTGG TACGCCGGAG AAGCGGGCGT GGCTGCGCGA GCAGGGGATC GCGCACGTGA
36241 TGGACTCGCG GTCGCTGGAC TTCGCCGAGC AAGTGCTGGC CGCGACGAAG GGCGAGGGGG
36301 TCGACGTCGT GTTGAACTCG CTGTCTGGCG CCGCGATCGA CGCGAGCCTT GCGACCCTCG
36361 TGCCGGACGG CCGCTTCATC GAGCTCGGCA AGACGGACAT CTATGCAGAT CGCTCGCTGG
36421 GGCTCGCTCA CTTTAGGAAG AGCCTGTCCT ACAGCGCCGT CGATCTTGCG GGTTTGGCCG
36481 TGCGTCGGCC CGAGCGCGTC GCAGCGCTGC TGGCGGAGGT GGTGGACCTG CTCGCACGGG
36541 GAGCGCTGCA GCCGCTTCCG GTAGAGATCT TCCCCCTCTC GCGGGCCGCG GACGCGTTCC
36601 GGAAAATGGC GCAAGCGCAG CATCTCGGGA AGCTCGTGCT CGCGCTGGAG GACCCGGACG
36661 TGCGGATCCG CGTTCCGGGC GAATCCGGCG TCGCCATCCG CGCGGACGGC ACCTACCTCG
36721 TGACCGGCGG TCTGGGTGGG CTCGGTCTGA GCGTGGCTGG ATGGCTGGCC GAGCAGGGGG
36781 CTGGGCATCT GGTGCTGGTG GGCCGCTCCG GTGCGGTGAG CGCGGAGCAG CAGACGGCTG
36841 TCGCCGCGCT CGAGGCGCAC GGCGCGCGTG TCACGGTAGC GAGGGCAGAC GTCGCCGATC
36901 GGGCGCAGAT CGAGCGGATC CTCCGCGAGG TTACCGCGTC GGGGATGCCG CTCCGCGGCG
36961 TCGTTCATGC GGCCGGTATC CTGGACGACG GGCTGCTGAT GCAGCAAACC CCCGCGCGGT
37021 TCCGCGCGGT CATGGCGCCC AAGGTCCGAG GGGCCTTGCA CCTGCATGCG TTGACACGCG
37081 AAGCGCCGCT CTCCTTCTTC GTGCTGTACG CTTCGGGAGC AGGGCTCTTG GGCTCGCCGG
37141 GCCAGGGCAA CTACGCCGCG GCCAACACGT TCCTCGACGC TCTGGCACAC CACCGGAGGG
```

37201 CGCAGGGGCT GCCAGCATTG AGCATCGACT GGGGCCTGTT CGCGGACGTG GGTTTGGCCG
37261 CCGGGCAGCA AAATCGCGGC GCACGGCTGG TCACCCGCGG GACGCGGAGC CTCACCCCCG
37321 ACGAAGGGCT GTGGGCGCTC GAGCGTCTGC TCGACGGCGA TCGCACCCAG GCCGGGGTCA
37381 TGCCGTTCGA CGTGCGGCAG TGGGTGGAGT TCTACCCGGC GGCGGCATCT TCGCGGAGGT
37441 TGTCGCGGCT GGTGACGGCA CGGCGCGTGG CTTCCGGTCG GCTCGCCGGG GATCGGGACC
37501 TGCTCGAACG GCTCGCCACC GCCGAGGCGG GCGCGCGGGC AGGAATGCTG CAGGAGGTCG
37561 TGCGCGCGCA GGTCTCGCAG GTGCTGCGCC TCCCCGAAGG CAAGCTCGAC GTGGATGCGC
37621 CGCTCACGAG CCTGGGAATG GACTCGCTGA TGGGGCTAGA GCTGCGCAAC CGCATCGAGG
37681 CCGTGCTCGG CATCACCATG CCGGCGACCC TGCTGTGGAC CTACCCCACG GTGGCAGCGC
37741 TGAGTGCGCA TCTGGCTTCT CATGTCGTCT CTACGGGGGA TGGGGAATCC GCGCGCCCGC
37801 CGGATACAGG GAACGTGGCT CCAATGACCC ACGAAGTCGC TTCGCTCGAC GAAGACGGGT
37861 TGTTCGCGTT GATTGATGAG TCACTCGCGC GTGCGGGAAA GAGGTGATTG CGTGACAGAC
37921 CGAGAAGGCC AGCTCCTGGA GCGCTTGCGT GAGGTTACTC TGGCCCTTCG CAAGACGCTG
37981 AACGAGCGCG ATACCCTGGA GCTCGAGAAG ACCGAGCCGA TCGCCATCGT GGGGATCGGC
38041 TGCCGCTTCC CCGGCGGAGC GGGCACTCCG GAGGCGTTCT GGGAGCTGCT CGACGACGGG
38101 CGCGACGCGA TCCGGCCGCT CGAGGAGCGC TGGGCGCTCG TAGGTGTCGA CCCAGGCGAC
38161 GACGTACCGC GCTGGGCGGG GCTGCTCACC GAAGCCATCG ACGGCTTCGA CGCCGCGTTC
38221 TTCGGTATCG CCCCCCGGGA GGCACGGTCG CTCGACCCGC AGCATCGCTT GCTGCTGGAG
38281 GTCGCCTGGG AGGGGTTCGA AGACGCCGGC ATCCCGCCTA GGTCCCTCGT CGGGAGCCGC
38341 ACCGGCGTGT TCGTCGGCGT CTGCGCCACG GAGTATCTCC ACGCCGCCGT CGCGCACCAG
38401 CCGCGCGAAG AGCGGGACGC GTACAGCACC ACCGGCAACA TGCTCAGCAT CGCCGCCGGA
38461 CGGCTATCGT ACACGCTGGG GCTGCAGGGA CCTTGCCTGA CCGTCGACAC GGCGTGCTCG
38521 TCATCGCTGG TGGCCATTCA CCTCGCCTGC CGCAGCCTGC GCGCTCGAGA GAGCGATCTC
38581 GCGCTGGCGG GAGGGGTCAA CATGCTTCTC TCCCCCGACA CGATGCGAGC TCTGGCGCGC
38641 ACCCAGGCGC TGTCGCCCAA TGGCCGTTGC CAGACCTTCG ACGCGTCGGC CAACGGGTTC
38701 GTCCGTGGGG AGGGCTGCGG TCTGATCGTG CTCAAGCGAT TGAGCGACGC GCGGCGGGAT
38761 GGGGACCGGA TCTGGGCGCT GATCCGAGGA TCGGCCATCA ATCAGGACGG CCGGTCGACG
38821 GGGTTGACGG CGCCCAACGT GCTCGCCCAG GGGGCGCTCT TGCGCGAGGC GCTGCGGAAC
38881 GCCGGCGTCG AGGCCGAGGC CATCGGTTAC ATCGAGACCC ACGGGGCGGC GACCTCGCTG
38941 GGCGACCCCA TCGAGATCGA AGCGCTGCGC ACCGTGGTGG GGCCGGCGCG AGCCGACGGA
39001 GCGCGCTGCG TGCTGGGCGC GGTGAAGACC AACCTCGGCC ACCTGGAGGG CGCTGCCGGC
39061 GTGGCGGGCC TGATCAAGGC TACACTTTCG CTACATCACG AGCGCATCCC GAGGAACCTC
39121 AACTTTCGTA CGCTCAATCC GCGGATCCGG ATCGAGGGGA CCGCGCTCGC GTTGGCGACC
39181 GAACCGGTGC CCTGGCCGCG GACGGGCCGG ACGCGCTTCG CGGGAGTGAG CTCGTTCGGG
39241 ATGAGCGGGA CCAACGCGCA TGTGGTGTTG GAGGAGGCGC CGGCGGTGGA GCCTGAGGCC
39301 GCGGCCCCCG AGCGCGCTGC GGAGCTGTTC GTCCTGTCGG CGAAGAGCGT GGCGGCGCTG
39361 GATGCGCAGG CAGCCCGGCT GCGGGACCAC CTGGAGAAGC ATGTCGAGCT TGGCCTCGGC
39421 GATGTGGCGT TCAGCCTGGC GACGACGCGC AGCGCGATGG AGCACCGGCT GGCGGTGGCC
39481 GCGAGCTCGC GCGAGGCGCT GCGAGGGGCG CTTTCGGCCG CAGCGCAGGG GCATACGCCG
39541 CCGGGAGCCG TGCGTGGGCG GGCCTCCGGC GGCAGCGCGC CGAAGGTGGT CTTCGTGTTT
39601 CCCGGCCAGG GCTCGCAGTG GGTGGGCATG GGCCGAAAGC TCATGGCCGA AGAGCCGGTC
39661 TTCCGGGCGG CGCTGGAGGG TTGCGACCGG GCCATCGAGG CGGAAGCGGG CTGGTCGCTG
39721 CTCGGGGAGC TCTCCGCCGA CGAGGCCGCC TCGCAGCTCG GGCGCATCGA CGTGGTTCAG
39781 CCGGTGCTCT TCGCCATGGA AGTAGCGCTT TCTGCGCTGT GGCGGTCGTG GGAGTGGAG
39841 CCGGAAGCGG TGGTGGGCCA CAGCATGGGC GAGGTGGCGG CGGCGCACGT GGCCGGCGCG
39901 CTGTCGCTCG AGGACGCGGT GGCGATCATC TGCCGGCGCA GCCGGCTGCT GCGGCGGATC
39961 AGCGGTCAGG GCGAGATGGC GCTGGTCGAG CTGTCGCTGG AGGAGGCCGA GGCGGCGCTG
40021 CGTGGCCATG AGGGTCGGCT GAGCGTGGCG GTGAGCAACA GCCCGCGCTC GACCGTGCTC
40081 GCAGGCGAGC CGGCGGCGCT CTCGGAGGTG CTGGCGGCGC TGACGGCCAA GGGGGTGTTC
40141 TGGCGGCAGG TGAAGGTGGA CGTCGCCAGC CATAGCCCGC AGGTCGACCC GCTGCGCGAA
40201 GAGCTGATCG CGGCGCTGGG GGCGATCCGG CCGCGAGCGG CTGCGGTGCC GATGCGCTCG
40261 ACGGTGACGG GCGGGGTGAT CGCGGGTCCG GAGCTCGGTG CGAGCTACTG GGCGGACAAT
40321 CTTCGGCAGC CGGTGCGCTT CGCTGCGGCG GCGCAAGCGC TGCTGGAAGG TGGCCCCACG
40381 CTGTTCATCG AGATGAGCCC GCACCCGATC CTGGTGCCGC CCCTGGACGA GATCCAGACG

40441 GCGGTCGAGC AAGGGGGCGC TGCGGTGGGC TCGCTGCGGC GAGGGCAGGA CGAGCGCGCG
40501 ACGCTGCTGG AGGCGCTGGG GACGCTGTGG GCGTCCGGCT ATCCGGTGAG CTGGGCTCGG
40561 CTGTTCCCCG CGGGCGGCAG GCGGGTTCCG CTGCCGACCT ATCCCTGGCA GCACGAGCGG
40621 TGCTGGATCG AGGTCGAGCC TGACGCCCGC CGCCTCGCCG CAGCCGACCC CACCAAGGAC
40681 TGGTTCTACC GGACGGACTG GCCCGAGGTG CCCCGCGCCG CCCCGAAATC GGAGACAGCT
40741 CATGGGAGCT GGCTGCTGTT GGCCGACAGG GGTGGGGTCG GCGAGGCGGT CGCTGCAGCG
40801 CTGTCGACGC GCGGACTTTC CTGCACCGTG CTTCATGCGT CGGCTGACGC CTCCACCGTC
40861 GCCGAGCAGG TATCCGAAGC TGCCAGTCGC CGAAACGACT GGCAGGGAGT CCTCTACCTG
40921 TGGGGCCTCG ACGCCGTCGT CGATGCTGGG GCATCGGCCG ACGAAGTCAG CGAGGCTACC
40981 CGCCGTGCCA CCGCACCCGT CCTTGGGCTG GTTCGATTCC TGAGCGCTGC GCCCCATCCT
41041 CCTCGCTTCT GGGTGGTGAC CCGCGGGGCA TGCACGGTGG GCGGCGAGCC AGAGGTCTCT
41101 CTTTGCCAAG CGGCGTTGTG GGGCCTCGCG CGCGTCGTGG CGCTGGAGCA TCCCGCTGCC
41161 TGGGGTGGCC TCGTGGACCT GGATCCTCAG AAGAGCCCGA CGGAGATCGA GCCCCTGGTG
41221 GCCGAGCTGC TTTCGCCGGA CGCCGAGGAT CAACTGGCGT TCCGCAGCGG TCGCCGGCAC
41281 GCAGCACGCC TTGTAGCCGC CCCGCCGGAG GGCGACGTCG CACCGATATC GCTGTCCGCG
41341 GAGGGAAGCT ACCTGGTGAC GGGTGGGCTG GGTGGCCTTG GTCTGCTCGT GGCTCGGTGG
41401 CTGGTGGAGC GGGGAGCTCG ACATCTGGTG CTCACCAGCC GGCACGGGCT GCCAGAGCGA
41461 CAGGCGTCGG GCGGAGAGCA GCCGCCGGAG GCCCGCGCGC GCATCGCAGC GGTCGAGGGG
41521 CTGGAAGCGC AGGGCGCGCG GGTGACCGTG GCAGCGGTGG ATGTCGCCGA GGCCGATCCC
41581 ATGACGGCGC TGCTGGCCGC CATCGAGCCC CCGTTGCGCG GGGTGGTGCA CGCCGCCGGC
41641 GTCTTCCCCG TGCGTCCCCT GGCGGAGACG GACGAGGCCC TGCTGGAGTC GGTGCTCCGT
41701 CCCAAGGTGG CCGGGAGCTG GCTGCTGCAC CGGCTGCTGC GCGACCGGCC TCTCGACCTG
41761 TTCGTGCTGT TCTCGTCGGG CGCGGCGGTG TGGGGTGGCA AAGGCCAAGG CGCATACGCC
41821 GCGGCCAATG CGTTCCTCGA CGGGCTCGCG CACCATCGCC GCGCGCACTC CCTGCCGGCG
41881 TTGAGCCTCG CCTGGGGCCT ATGGGCCGAG GGAGGCGTGG TTGATGCAAA GGCTCATGCA
41941 CGTCTGAGCG ACATCGGAGT CCTGCCCATG GCCACGGGGC CGGCCTTGTC GGCGCTGGAG
42001 CGCCTGGTGA ACACCAGCGC TGTCCAGCGT TCGGTCACAC GGATGGACTG GCGCGCGCTTC
42061 GCGCCGGTCT ATGCCGCGCG AGGGCGGCGC AACTTGCTTT CGGCTCTGGT CGCGGAGGAC
42121 GAGCGCACTG CGTCTCCCCC GGTGCCGACG GCAAACCGGA TCTGGCGCGG CCTGTCCGTT
42181 GCGGAGAGCC GCTCAGCCCT CTACGAGCTC GTTCGCGGCA TCGTCGCCCG GGTGCTGGGC
42241 TTCTCCGACC CGGGCGCGCT CGACGTCGGC CGAGGCTTCG CCGAGCAGGG GCTCGACTCC
42301 CTGATGGCTC TGGAGATCCG TAACCGCCTT CAGCGCGAGC TGGGCGAACG GCTGTCGGCG
42361 ACTCTGGCCT TCGACCACCC GACGGTGGAG CGGCTGGTGG CGCATCTCCT CACCGACGTG
42421 CTGAAGCTGG AGGACCGGAG CGACACCCGG CACATCCGGT CGGTGGCGGC GGATGACGAC
42481 ATCGCCATCG TCGGTGCCGC CTGCCGGTTC CCGGGCGGGG ATGAGGGCCT GGAGACATAC
42541 TGGCGGCATC TGGCCGAGGG CATGGTGGTC AGCACCGAGG TGCCAGCCGA CCGGTGGCGC
42601 GCGGCGGACT GGTACGACCC CGATCCGGAG GTTCCGGGCC GGACCTATGT GGCCAAGGGG
42661 GCCTTCCTCC GCGATGTGCG CAGCTTGGAT GCGGCGTTCT TCTCCATCTC CCCTCGTGAG
42721 GCGATGAGCC TGGACCCGCA ACAGCGGCTG TTGCTGGAGG TGAGCTGGGA GGCGATCGAG
42781 CGCGCTGGCC AGGACCCGAT GGCGCTGCGC GAGAGCGCCA CGGGCGTGTT CGTGGGCATG
42841 ATCGGGAGCG AGCACGCCGA GCGGGTGCAG GGCCTCGACG ACGACGCGGC GTTGCTGTAC
42901 GGCACCACCG GCAACCTGCT CAGCGTCGCC GCTGGACGGC TGTCGTTCTT CCTGGGTCTG
42961 CACGGCCCGA CGATGACGGT GGACACCGCG TGCTCGTCGT CGCTGGTGGC GTTGCACCTC
43021 GCCTGCCAGA GCCTGCGATT GGGCGAGTGC GACCAGGCAC TGGCCGGCGG GTCCAGCGTG
43081 CTTTTGTCGC CGCGGTCATT CGTCGCGGCA TCGCGCATGC GTTTGCTTTC GCCAGATGGG
43141 CGGTGCAAGA CGTTCTCGGC CGCTGCAGAC GGCTTTGCGC GGGCCGAGGG CTGCGCCGTG
43201 GTGGTGCTCA AGCGGCTCCG TGACGCGCAG CGCGACCGCG ACCCCATCCT GGCGGTGGTC
43261 CGGAGCACGG CGATCAACCA CGATGGCCCG AGCAGCGGGC TCACGGTGCC CAGCGGTCCT
43321 GCCCAGCAGG CGTTGCTAGG CCAGGCGCTG GCGCAAGCGG GCGTGGCACC GGCCGAGGTC
43381 GATTTCGTGG AGTGCCACGG GACGGGGACA GCGCTGGGTG ACCCGATCGA GGTGCAGGCG
43441 CTGGGCGCGG TGTATGGCCG GGGCCGCCCC GCGGAGCGGC CGCTCTGGCT GGGCGCTGTC
43501 AAGGCCAACC TCGGCCACCT GGAGGCCGCG GCGGGCTTGG CCGGCGTGCT CAAGGTGCTC
43561 TTGGCGCTGG AGCACGAGCA GATTCCGGCT CAACCGGAGC TCGACGAGCT CAACCCGCAC
43621 ATCCCGTGGG CAGAGCTGCC AGTGGCCGTT GTCCGCGCGG CGGTCCCCTG GCCGCGCGGC

43681 GCGCGCCCGC GTCGTGCAGG CGTGAGCGCT TTCGGCCTGA GCGGGACCAA CGCGCATGTG
43741 GTGTTGGAGG AGGCGCCGGC GGTGGAGCCT GAGGCCGCGG CCCCCGAGCG CGCTGCGGAG
43801 CTGTTCGTCC TGTCGGCGAA GAGCGTGGCG GCGCTGGATG CGCAGGCAGC CCGGCTGCGG
43861 GATCATCTGG AGAAGCATGT CGAGCTTGGC CTCGGCGATG TGGCGTTCAG CCTGGCGACG
43921 ACGCGCAGCG CGATGGAGCA CCGGCTGGCG GTGGCCGCGA GCTCGCGCGA GGCGCTGCGA
43981 GGGGCGCTTT CGGCCGCAGC GCAGGGGCAT ACGCCGCCGG GAGCCGTGCG TGGGCGGGCC
44041 TCCGGCGGCA GCGCGCCGAA GGTGGTCTTC GTGTTTCCCG GCCAGGGCTC GCAGTGGGTG
44101 GGCATGGGCC GAAAGCTCAT GGCCGAAGAG CCGGTCTTCC GGGCGGCGCT GGAGGGTTGC
44161 GACCGGGCCA TCGAGGCGGA AGCGGGCTGG TCGCTGCTCG GGAGCTCTCC CGCCGACGAG
44221 GCCGCCTCGC AGCTCGGGCG CATCGACGTG GTTCAGCCGG TGCTCTTCGC CGTGGAAGTA
44281 GCGCTTTCAG CGCTGTGGCG GTCGTGGGGA GTGGAGCCGG AAGCGGTGGT GGGCCACAGC
44341 ATGGGCGAGG TTGCGGCGGC GCACGTGGCC GGCGCGCTGT CGCTCGAGGA TGCGGTGGCG
44401 ATCATCTGCC GGCGCAGCCG GCTGCTGCGG CGGATCAGCG GTCAGGGCGA GATGGCGCTG
44461 GTCGAGCTGT CGCTGGAGGA GGCCGAGGCG GCGCTGCGTG GCCATGAGGG TCGGCTGAGC
44521 GTGGCGGTGA GCAACAGCCC GCGCTCGACC GTGCTCGCAG GCGAGCCGGC GGCGCTCTCG
44581 GAGGTGCTGG CGGCGCTGAC GGCCAAGGGG GTGTTCTGGC GGCAGGTGAA GGTGGACGTC
44641 GCCAGCCATA GCCCGCAGGT CGACCCGCTG CGCGAAGAGC TGGTCGCGGC GCTGGGAGCG
44701 ATCCGGCCGC GAGCGGCTGC GGTGCCGATG CGCTCGACGG TGACGGGCGG GGTGATTGCG
44761 GGTCCGGAGC TCGGTGCGAG CTACTGGGCG GACAATCTTC GGCAGCCGGT GCGCTTCGCT
44821 GCGGCGGCGC AAGCGCTGCT GGAAGGTGGC CCCACGCTGT TCATCGAGAT GAGCCCGCAC
44881 CCGATCCTGG TGCCGCCTCT GGACGAGATC CAGACGGCGG TCGAGCAAGG GGGCGCTGCG
44941 GTGGGCTCGC TGCGGCGAGG GCAGGACGAG CGCGCGACGC TGCTGGAGGC GCTGGGGACG
45001 CTGTGGGCGT CCGGCTATCC GGTGAGCTGG GCTCGGCTGT TCCCCGCGGG CGGCAGGCGG
45061 GTTCCGCTGC CGACCTATCC CTGGCAGCAC GAGCGGTACT GGATCGAGGA CAGCGTGCAT
45121 GGGTCGAAGC CCTCGCTGCG GCTTCGGCAG CTTCATAACG GCGCCACGGA CCATCCGCTG
45181 CTCGGGGCTC CATTGCTCGT CTCGGCGCGA CCCGGAGCTC ACTTGTGGGA GCAAGCGCTG
45241 AGCGACGAGA GGCTATCCTA TCTTTCGGAA CATAGGGTCC ATGGCGAAGC CGTGTTGCCC
45301 AGCGCGGCGT ATGTAGAGAT GGCGCTCGCC GCCGGCGTAG ATCTCTATGG CGCGGCGACG
45361 CTGGTGCTGG AGCAGCTGGC GCTCGAGCGA GCCCTCGCCG TGCCTTCCGA AGGCGGACGC
45421 ATCGTGCAAG TGGCCCTCAG CGAAGAAGGG CCCGGTCGGG CCTCATTCCA GGTATCGAGC
45481 CGTGAGGAGG CAGGTAGAAG CTGGGTTCGG CACGCCACGG GGCACGTGTG TAGCGACCAG
45541 AGCTCAGCAG TGGGAGCGTT GAAGGAAGCT CCGTGGGAGA TTCAACAGCG ATGTCCGAGC
45601 GTCCTGTCGT CGGAGGCGCT CTATCCGCTG CTCAACGAGC ACGCCCTCGA CTATGGCCCC
45661 TGCTTCCAGG GTGTGGAGCA GGTGTGGCTC GGCACGGGGG AGGTGCTCGG CCGGGTACGC
45721 TTGCCAGAAG ACATGGCATC CTCAAGTGGC GCCTATCGGA TTCATCCCGC CTTGTTGGAT
45781 GCATGTTTTC AAGTGCTGAC CGCGCTGCTC ACCACGCCGG AATCCATCGA GATTCGGAGG
45841 CGGCTGACGG ATCTCCACGA ACCGGATCTC CCGCGGTCCA GGGCTCCGGT GAATCAAGCG
45901 GTGAGTGACA CCTGGCTGTG GGACGCCGCG CTGGACGGTG GACGGCGCCA GAGCGCGAGC
45961 GTGCCCGTCG ACCTGGTGCT CGGCAGCTTC CACGCGAAGT GGGAGGTCAT GGATCGCCTC
46021 GCGCAGACGT ACATCATCCG CACTCTCCGC ACATGGAACG TCTTCTGCGC TGCTGGAGAG
46081 CGTCACACGA TAGACGAGTT GCTCGTCAGG CTCCAAATCT CTGCTGTCTA CAGGAAGGTC
46141 ATCAAGCGAT GGATGGATCA CCTTGTCGCG ATCGGCGTCC TTGTAGGGGA CGGAGAGCAT
46201 CTTGTGAGCT CTCAGCCGCT GCCGGAGCAT GATTGGGCGG CGGTGCTCGA GGAGGCCGCG
46261 ACGGTGTTCG CCGACCTCCC AGTCCTACTT GAGTGGTGCA AGTTTGCCGG GGAACGGCTC
46321 GCGGACGTGT TGACCGGGAA GACGCTGGCG CTCGAGATCC TCTTCCCTGG CGGCTCGTTC
46381 GATATGGCGG AGCGAATCTA TCAAGATTCG CCCATCGCCC GTTACTCGAA CGGCATCGTG
46441 CGCGGTGTCG TCGAGTCGGC GGCGCGGGTG GTAGCACCGT CGGGAACGTT CAGCATCTTG
46501 GAGATCGGAG CAGGGACGGG CGCGACCACC GCCGCCGTCC TCCCGGTGTT GCTGCCTGAC
46561 CGGACAGAAT ACCATTTCAC CGATGTTTCT CCGCTCTTCC TTGCTCGTGC GGAGCAAAGA
46621 TTTCGAGATC ATCCATTCCT GAAGTATGGT ATTCTGGATA TCGACCAGGA GCCAGCTGGC
46681 CAGGGATACG CACATCAGAA GTTCGACGTC ATCGTCGCGG CCAACGTCAT CCATGCGACC
46741 CGCGATATAA GAGCCACGGC GAAGCGTCTC CTGTCGTTGC TCGCGCCCGG AGGCCTTCTG
46801 GTGCTGGTCG AGGGCACAGG GCATCCGATC TGGTTCGATA TCACCACGGG ATTGATCGAG
46861 GGGTGGCAGA AGTACGAAGA TGATCTTCGT ACCGACCATC CGCTCCTGCC TGCTCGGACC

```
46921 TGGTGTGACG TCCTGCGCCG GGTAGGCTTT GCGGATGCCG TGAGTCTGCC AGGCGACGGA
46981 TCTCCGGCGG GGATCCTCGG ACAGCACGTG ATCCTCTCGC GCGCTCCGGG CATAGCAGGA
47041 GCCGCTTGTG ACAGCTCCGG TGAGTCGGCG ACCGAATCGC CGGCCGCGCG TGCAGTACGG
47101 CAGGAATGGG CCGATGGCTC CGCTGACGGC GTCCATCGGA TGGCGTTGGA GAGAATGTAC
47161 TTCCACCGCC GGCCGGGCCG GCAGGTTTGG GTCCACGGTC GATTGCGTAC CGGTGGAGGC
47221 GCGTTCACGA AGGCGCTCAC TGGAGATCTG CTCCTGTTCG AAGAGACCGG GCAGGTCGTG
47281 GCAGAGGTTC AGGGGCTCCG CCTGCCGCAG CTCGAGGCTT CTGCTTTCGC GCCGCGGGAC
47341 CCGCGGGAAG AGTGGTTGTA CGCGTTGGAA TGGCAGCGCA AAGACCCTAT ACCAGAGGCT
47401 CCGGCAGCCG CGTCTTCTTC CACCGCGGGG GCTTGGCTCG TGCTGATGGA CCAGGGCGGG
47461 ACAGGCGCTG CGCTCGTATC GCTGCTGGAA GGGCGAGGCG AGGCGTGCGT GCGCGTCGTC
47521 GCGGGTACGG CATACGCCTG CCTCGCGCCG GGGCTGTATC AAGTCGATCC GGCGCAGCCA
47581 GATGGCTTTC ATACCCTGCT CCGCGATGCA TTCGGCGAGG ACCGGATGTG CCGCGCGGTA
47641 GTGCATATGT GGAGCCTTGA TGCGAAGGCA GCAGGGGAGA GGACGACAGC GGAGTCGCTT
47701 CAGGCCGATC AACTCCTGGG GAGCCTGAGC GCGCTTTCTC TGGTGCAGGC GCTGGTGCGC
47761 CGGAGGTGGC GCAACATGCC GCGACTTTGG CTCTTGACCC GCGCCGTGCA TGCGGTGGGC
47821 GCGGAGGACG CAGCGGCCTC GGTGGCGCAG GCGCCGGTGT GGGGCCTCGG TCGGACGCTC
47881 GCGCTCGAGC ATCCAGAGCT GCGGTGCACG CTCGTGGACG TGAACCCGGC GCCGTCTCCA
47941 GAGGACGCAG CTGCACTCGC GGTGGAGCTC GGGGCGAGCG ACAGAGAGGA CCAGATCGCA
48001 TTGCGCTCGA ATGGCCGCTA CGTGGCGCGC CTCGTGCGGA GCTCCTTTTC CGGCAAGCCT
48061 GCTACGGATT GCGGCATCCG GGCGGACGGC AGTTATGTGA TCACCGATGG CATGGGGAGA
48121 GTGGGGCTCT CGGTCGCGCA ATGGATGGTG ATGCAGGGGG CCCGCCATGT GGTGCTCGTG
48181 GATCGCGGCG GCGCTTCCGA CGCCTCCCGG GATGCCCTCC GGTCCATGGC CGAGGCTGGC
48241 GCAGAGGTGC AGATCGTGGA GGCCGACGTG GCTCGGCGCG TCGATGTCGC TCGGCTTCTC
48301 TCGAAGATCG AACCGTCGAT GCCGCCGCTT CGGGGGATCG TGTACGTGGA CGGGACCTTC
48361 CAGGGCGACT CCTCGATGCT GGAGCTGGAT GCCCATCGCT TCAAGGAGTG GATGTATCCC
48421 AAGGTGCTCG GAGCGTGGAA CCTGCACGCG CTGACCAGGG ATAGATCGCT GGACTTCTTC
48481 GTCCTGTACT CCTCGGGCAC CTCGCTTCTG GGCTTGCCCG GACAGGGGAG CCGCGCCGCC
48541 GGTGACGCCT TCTTGGACGC CATCGCGCAT CACCGGTGTA GGCTGGGCCT CACAGCGATG
48601 AGCATCAACT GGGGATTGCT CTCCGAAGCA TCATCGCCGG CGACCCCGAA CGACGGCGGC
48661 GCACGGCTCC AATACCGGGG GATGGAAGGT CTCACGCTGG AGCAGGGAGC GGAGGCGCTC
48721 GGGCGCTTGC TCGCACAACC CAGGGCGCAG GTAGGGGTAA TGCGGCTGAA TCTGCGCCAG
48781 TGGCTGGAGT TCTATCCCAA CGCGGCCCGA CTGGCGCTGT GGGCGGAGTT GCTGAAGGAG
48841 CGTGACCGCA CCGACCGGAG CGCGTCGAAC GCATCGAACC TGCGCGAGGC GCTGCAGAGC
48901 GCCAGGCCCG AAGATCGTCA GTTGGTTCTG GAGAAGCACT TGAGCGAGCT GTTGGGGCGG
48961 GGGCTGCGCC TTCCGCCGGA GAGGATCGAG CGGCACGTGC CGTTCAGCAA TCTCGGCATG
49021 GACTCGTTGA TAGGCCTGGA GCTCCGCAAC CGCATCGAGG CCGCGCTCGG CATCACCGTG
49081 CCGGCGACCC TGCTATGGAC TTACCCTACC GTAGCAGCTC TGAGCGGGAA CCTGCTAGAT
49141 ATTCTGTTCC CGAATGCCGG CGCGACTCAC GCTCCGGCCA CCGAGCGGGA GAAGAGCTTC
49201 GAGAACGATG CCGCAGATCT CGAGGCTCTG CGGGGTATGA CGGACGAGCA GAAGGACGCG
49261 TTGCTCGCCG AAAAGCTGGC GCAGCTCGCG CAGATCGTTG GTGAGTAAGG GACTGAGGGA
49321 GTATGGCGAC CACGAATGCC GGGAAGCTTG AGCATGCCCT TCTGCTCATG GACAAGCTTG
49381 CGAAAAAGAA CGCGTCTTTG GAGCAAGAGC GGACCGAGCC GATCGCCATC ATAGGTATTG
49441 GCTGCCGCTT CCCCGGCGGA GCGGACACTC CGGAGGCATT CTGGGAGCTG CTCGACTCGG
49501 GCCGAGACGC GGTCCAGCCG CTCGACCGGC GCTGGGCGCT GGTCGGCGTC CATCCCAGCG
49561 AGGAGGTGCC GCGCTGGGCC GGACTGCTCA CCGAGGCGGT GGACGGCTTC GACGCCGCGT
49621 TCTTTGGCAC CTCGCCTCGG GAGGCGCGGT CGCTCGATCC TCAGCAACGC CTGCTGCTGG
49681 AGGTCACCTG GGAAGGGCTC GAGGACGCCG GCATCGCACC CCAGTCCCTC GACGGCAGCC
49741 GCACCGGGGT ATTCCTGGGC GCATGCAGCA GCGACTACTC GCATACCGTT GCGCAACAGC
49801 GGCGCGAGGA GCAGGACGCG TACGACATCA CCGGCAATAC GCTCAGCGTC GCCGCCGGAC
49861 GGTTGTCTTA TACGCTAGGG CTGCAGGGAC CCTGCCTGAC CGTCGACACG GCCTGCTCGT
49921 CGTCGCTCGT GGCCATCCAC CTTGCCTGCC GCAGCCTGCG CGCTCGCGAG AGCGATCTCG
49981 CGCTGGCGGG GGGCGTCAAC ATGCTCCTTT CGTCCAAGAC GATGATAATG CTGGGGCGCA
50041 TCCAGGCGCT GTCGCCCGAT GGCCACTGCC GGACATTCGA CGCCTCGGCC AACGGGTTCG
50101 TCCGTGGGGA GGGCTGCGGT ATGGTCGTGC TCAAACGGCT CTCCGACGCC CAGCGACATG
```

50161 GCGATCGGAT CTGGGCTCTG ATCCGGGGTT CGGCCATGAA TCAGGATGGC CGGTCGACAG
50221 GGTTGATGGC ACCCAATGTG CTCGCTCAGG AGGCGCTCTT ACGCCAGGCG CTGCAGAGCG
50281 CTCGCGTCGA CGCCGGGGCC ATCGATTATG TCGAGACCCA CGGAACGGGG ACCTCGCTCG
50341 GCGACCCGAT CGAGGTCGAT GCGCTGCGTG CCGTGATGGG GCCGGCGCGG GCCGATGGGA
50401 GCCGCTGCGT GCTGGGCGCA GTGAAGACCA ACCTCGGCCA CCTGGAGGGC GCTGCAGGCG
50461 TGGCGGGTTT GATCAAGGCG GCGCTGGCTC TGCACCACGA ATCGATCCCG CGAAACCTCC
50521 ATTTTCACAC GCTCAATCCG CGGATCCGGA TCGAGGGGAC CGCGCTCGCG CTGGCGACGG
50581 AGCCGGTGCC GTGGCCGCGG GCGGGCCGAC CGCGCTTCGC GGGGGTGAGC GCGTTCGGCC
50641 TCAGCGGCAC CAACGTCCAT GTCGTGCTGG AGGAGGCGCC GGCCACGGTG CTCGCACCGG
50701 CGACGCCGGG GCGCTCAGCA GAGCTTTTGG TGCTGTCGGC GAAGAGCACC GCCGCGCTGG
50761 ACGCACAGGC GGCGCGGCTC TCAGCGCACA TCGCCGCGTA CCCGGAGCAG GGCCTCGGAG
50821 ACGTCGCGTT CAGCCTGGTA GCGACGCGGA GCCCGATGGA GCACCGGCTC GCGGTGGCGG
50881 CGACCTCGCG CGAGGCGCTG CGAAGCGCGC TGGAAGCTGC GGCGCAGGGG CAGACCCCGG
50941 CAGGCGCGGC GCGCGGCAGG GCCGCTTCCT CGCCCGGCAA GCTCGCCTTC CTGTTCGCCG
51001 GGCAGGGCGC GCAGGTGCCG GGCATGGGCC GTGGGTTGTG GGAGGCGTGG CCGGCGTTCC
51061 GCGAGACCTT CGACCGGTGC GTCACGCTCT TCGACCGGGA GCTCCATCAG CCGCTCTGCG
51121 AGGTGATGTG GGCCGAGCCG GGCAGCAGCA GGTCGTCGTT GCTGGACCAG ACGGCATTCA
51181 CCCAGCCGGC GCTCTTTGCG CTGGAGTACG CGCTGGCCGC GCTCTTCCGG TCGTGGGGCG
51241 TGGAGCCGGA GCTCATCGCT GGCCATAGCC TCGGCGAGCT GGTGGCCGCC TGCGTGGCGG
51301 GTGTGTTCTC CCTCGAGGAC GCCGTGCGCT TGGTGGTCGC GCGCGGCCGG TTGATGCAGG
51361 CGCTGCCGGC CGGCGGTGCG ATGGTATCGA TCGCCGCGCC GGAGGCCGAC GTGGCTGCCG
51421 CGGTGGCGCC GCACGCAGCG TCGGTGTCGA TCGCGGCAGT CAATGGGCCG GAGCAGGTGG
51481 TGATCGCGGG CGCCGAGAAA TTCGTGCAGC AGATCGCGGC GGCGTTCGCG GCGCGGGGGG
51541 CGCGAACCAA ACCGCTGCAT GTTTCGCACG CGTTCCACTC GCCGCTCATG GATCCGATGC
51601 TGGAGGCGTT CCGGCGGGTG ACCGAGTCGG TGACGTATCG GCGGCCTTCG ATGGCGCTGG
51661 TGAGCAACCT GAGCGGGAAG CCCTGCACGG ATGAGGTGTG CGCGCCGGGT TACTGGGTGC
51721 GTCACGCGCG AGAGGCGGTG CGCTTCGCGG ACGGCGTGAA GGCGCTGCAC GCGGCCGGTG
51781 CGGGCATCTT CGTCGAGGTG GGCCCGAAGC CGGCGCTGCT CGGCCTTTTG CCGGCCTGCC
51841 TGCCGGATGC CAGGCCGGTG CTGCTCCCAG CGTCGCGCGC CGGGCGTGAC GAGGCTGCGA
51901 GCGCGCTGGA GGCGCTGGGT GGGTTCTGGG TCGTCGGTGG ATCGGTCACC TGGTCGGGTG
51961 TCTTCCCTTC GGGCGGACGG CGGGTACCGC TGCCAACCTA TCCCTGGCAG CGCGAGCGTT
52021 ACTGGATCGA AGCGCCGGTC GATGGTGAGG CGGACGGCAT CGGCCGTGCT CAGGCGGGGG
52081 ACCACCCCCT TCTGGGTGAA GCCTTTTCCG TGTCGACCCA TGCCGGTCTG CGCCTGTGGG
52141 AGACGACGCT GGACCGAAAG CGGCTGCCGT GGCTCGGCGA GCACCGGGCG CAGGGGGAGG
52201 TCGTGTTTCC TGGCGCCGGG TACCTGGAGA TGGCGCTGTC GTCGGGGGCC GAGATCTTGG
52261 GCGATGGACC GATCCAGGTC ACGGATGTGG TGCTCATCGA GACGCTGACC TTCGCGGGCG
52321 ATACGGCGGT ACCGGTCCAG GTGGTGACGA CCGAGGAGCG ACCGGGACGG CTGCGGTTCC
52381 AGGTAGCGAG TCGGGAGCCG GGGGCACGTC GCGCGTCCTT CCGGATCCAC GCCCGCGGCG
52441 TGCTGCGCCG GGTCGGGCGC GCCGAGACCC CGGCGAGGTT GAACCTCGCC GCCCTGCGCG
52501 CCCGGCTTCA TGCCGCCGTG CCCGCTGCGG CTATCTATGG GGCGCTCGCC GAGATGGGGC
52561 TTCAATACGG CCCGGCGTTG CGGGGGCTCG CCGAGCTGTG GCGGGGTGAG GGCGAGGCGC
52621 TGGGCAGAGT GAGACTGCCT GAGTCCGCCG GCTCCGCGAC AGCCTACCAG CTGCATCCGG
52681 TGCTGCTGGA CGCGTGCGTC CAAATGATTG TTGGCGCGTT CGCCGATCGC GATGAGGCGA
52741 CGCCGTGGGC GCCGGTGGAG GTGGGCTCGG TGCGGCTGTT CCAGCGGTCT CCTGGGGAGC
52801 TATGGTGCCA TGCGCGCGTC GTGAGCGATG GTCAACAGGC CCCCAGCCGG TGGAGCGCCG
52861 ACTTTGAGTT GATGGACGGT ACGGGCGCGG TGGTCGCCGA GATCTCCCGG CTGGTGGTGG
52921 AGCGGCTTGC GAGCGGTGTA CGCCGGCGCG ACGCAGACGA CTGGTTCCTG GAGCTGGATT
52981 GGGAGCCCGC GGCGCTCGAG GGGCCCAAGA TCACAGCCGG CCGGTGGCTG CTGCTCGGCG
53041 AGGGTGGTGG GCTCGGGCGC TCGTTGTGCT CAGCGCTGAA GGCCGCCGGC CATGTCGTCG
53101 TCCACGCCGC GGGGGACGAC ACGAGCGCTG CAGGAATGCG CGCGCTCCTG GCCAACGCGT
53161 TCGACGGCCA GGCCCCGACG GCCGTGGTGC ACCTCAGCAG CCTCGACGGG GGCGGCCAGC
53221 TCGACCCGGG GCTCGGGGCG CAGGGCGCGC TCGACGCGCC CCGGAGCCCA GATGTCGATG
53281 CCGATGCCCT CGAGTCGGCG CTGATGCGTG GTTGCGACAG CGTGCTCTCC CTGGTGCAAG
53341 CGCTGGTCGG CATGGACCTC CGAAATGCGC CGCGGCTGTG GCTTTTGACC CGCGGGGCTC

53401	AGGCGGCCGC	CGCCGGCGAT	GTCTCCGTGG	TGCAAGCGCC	GCTGTTGGGG	CTGGGCCGCA
53461	CCATCGCCTT	GGAGCACGCC	GAGCTGCGCT	GTATCAGCGT	CGACCTCGAT	CCAGCCCAGC
53521	CTGAAGGGGA	AGCCGATGCT	TTGCTGGCCG	AGCTACTTGC	AGATGATGCC	GAGGAGGAGG
53581	TCGCGCTGCG	CGGTGGCGAG	CGGTTTGTTG	CGCGGCTCGT	CCACCGGCTG	CCCGAGGCTC
53641	AACGCCGGGA	GAAGATCGCG	CCCGCCGGTG	ACAGGCCGTT	CCGGCTAGAG	ATCGATGAAC
53701	CCGGCGTGCT	GGACCAACTG	GTGCTCCGGG	CCACGGGGCG	GCGCGCTCCT	GGTCCGGGCG
53761	AGGTCGAGAT	CGCCGTCGAA	GCGGCGGGGC	TCGACTCCAT	CGACATCCAG	CTGGCGGTGG
53821	GCGTTGCTCC	CAATGACCTG	CCTGGAGGAG	AAATCGAGCC	GTCGGTGCTC	GGAAGCGAGT
53881	GCGCCGGGCG	CATCGTCGCT	GTGGGCGAGG	GCGTGAACGG	CCTTGTGGTG	GGCCAGCCGG
53941	TGATCGCCCT	TGCGGCGGGA	GTATTTGCTA	CCCATGTCAC	CACGTCGGCC	ACGCTGGTGT
54001	TGCCTCGGCC	TCTGGGGCTC	TCGGCGACCG	AGGCGGCCGC	GATGCCCCTC	GCGTATTTGA
54061	CGGCCTGGTA	CGCCCTCGAC	AAGGTCGCCC	ACCTGCAGGC	GGGGGAGCGG	GTGCTGATCC
54121	GTGCGGAGGC	CGGTGGTATC	GGTCTTTGCG	CGGTGCGATG	GGCGCAGCGC	GTGGGCGCCG
54181	AGGTGTATGC	GACCGCCGAC	ACGCCCGAGA	AACGTGCCTA	CCTGGAGTCG	CTGGGCGTGC
54241	GGTACGTGAG	CGATTCCCGC	TCGGGCCGGT	TCGCCGCAGA	CGTGCATGCA	TGGACGGACG
54301	GCGAGGGTGT	GGACGTCGTG	CTCGACTCGC	TTTCGGGCGA	GCACATCGAC	AAGAGCCTCA
54361	TGGTCCTGCG	CGCCTGTGGC	CGCCTTGTGA	AGCTGGGCAG	GCGCGACGAC	TGCGCCGACA
54421	CGCAGCCTGG	GCTGCCGCCG	CTCCTACGGA	ATTTTTCCTT	CTCGCAGGTG	GACTTGCGGG
54481	GAATGATGCT	CGATCAACCG	GCGAGGATCC	GTGCGCTCCT	CGACGAGCTG	TTCGGGTTGG
54541	TCGCAGCCGG	TGCCATCAGC	CCACTGGGGT	CGGGGTTGCG	CGTTGGCGGA	TCCCTCACGC
54601	CACCGCCGGT	CGAGACCTTC	CCGATCTCTC	GCGCAGCCGA	GGCATTCCGG	AGGATGGCGC
54661	AAGGACAGCA	TCTCGGGAAG	CTCGTGCTCA	CGCTGGACGA	CCCGGAGGTG	CGGATCCGCG
54721	CTCCGGCCGA	ATCCAGCGTC	GCCGTCCGCG	CGGACGGCAC	CTACCTTGTG	ACCGGCGGTC
54781	TGGGTGGGCT	CGGTCTGCGC	GTGGCCGGAT	GGCTGGCCGA	GCGGGGCGCG	GGGCAACTGG
54841	TGCTGGTGGG	CCGCTCCGGT	GCGGCGAGCG	CAGAGCAGCG	AGCCGCCGTG	GCGGCGCTAG
54901	AGGCCCACGG	CGCGCGCGTC	ACGGTGGCGA	AAGCGGATGT	CGCCGATCGG	TCACAGATCG
54961	AGCGGGTCCT	CCGCGAGGTT	ACCGCGTCGG	GGATGCCGCT	GCGGGGTGTC	GTGCATGCGG
55021	CAGGTCTTGT	GGATGACGGG	CTGCTGATGC	AGCAGACTCC	GGCGCGGCTC	CGCACGGTGA
55081	TGGGACCTAA	GGTCCAGGGA	GCCTTGCACT	TGCACACGCT	GACACGCGAA	GCGCCTCTTT
55141	CCTTCTTCGT	GCTGTACGCT	TCTGCAGCTG	GGCTGTTCGG	CTCGCCAGGC	CAGGGCAACT
55201	ATGCCGCAGC	CAACGCGTTC	CTCGACGCCC	TTTCGCATCA	CCGCAGGGCG	CACGGCCTGC
55261	CGGCGCTGAG	CATCGACTGG	GGCATGTTCA	CGGAGGTGGG	GATGGCCGTT	GCGCAAGAAA
55321	ACCGTGGCGC	GCGGCTGATC	TCTCGCGGGA	TGCGGGGCAT	CACCCCCGAT	GAGGGTCTGT
55381	CAGCTCTGGC	GCGCTTGCTC	GAGGGTGATC	GCGTGCAGAC	GGGGGTGATA	CCGATCACTC
55441	CGCGGCAGTG	GGTGGAGTTC	TACCCGGCAA	CAGCGGCCTC	ACGGAGGTTG	TCGCGGCTGG
55501	TGACCACGCA	GCGCGCGGTT	GCTGATCGGA	CCGCCGGGGA	TCGGGACCTG	CTCGAACAGC
55561	TTGCCTCGGC	TGAGCCGAGC	GCGCGGGCGG	GGCTGCTGCA	GGACGTCGTG	CGCGTGCAGG
55621	TCTCGCATGT	GCTGCGTCTC	CCTGAAGACA	AGATCGAGGT	GGATGCCCCG	CTCTCGAGCA
55681	TGGGCATGGA	CTCGCTGATG	AGCCTGGAGC	TGCGCAACCG	CATCGAGGCT	GCGCTGGGCG
55741	TCGCCGCGCC	TGCAGCCTTG	GGGTGGACGT	ACCCAACGGT	AGCAGCGATA	ACGCGCTGGC
55801	TGCTCGACGA	CGCCCTCGCC	GTCCGGCTTG	GCGGCGGGTC	GGACACGGAC	GAATCGACGG
55861	CAAGCGCCGG	ATCGTTCGTC	CACGTCCTCC	GCTTTCGTCC	TGTCGTCAAG	CCGCGGGCTC
55921	GTCTCTTCTG	TTTTCACGGT	TCTGGCGGCT	CGCCCGAGGG	CTTCCGTTCC	TGGTCGGAGA
55981	AGTCTGAGTG	GAGCGATCTG	GAAATCGTGG	CCATGTGGCA	CGATCGCAGC	CTCGCCTCCG
56041	AGGACGCGCC	TGGTAAGAAG	TACGTCCAAG	AGGCGGCCTC	GCTGATTCAG	CACTATGCAG
56101	ACGCACCGTT	TGCGTTAGTA	GGGTTCAGCC	TGGGTGTCCG	GTTCGTCATG	GGGACAGCCG
56161	TGGAGCTCGC	TAGTCGTTCC	GGCGCACCGG	CTCCGCTGGC	CGTTTTTGCG	TTGGGCGGCA
56221	GCTTGATCTC	TTCTTCAGAG	ATCACCCCGG	AGATGGAGAC	CGATATAATA	GCCAAGCTCT
56281	TCTTCCGAAA	TGCCGCGGGT	TTCGTGCGAT	CCACCCAACA	AGTTCAGGCC	GATGCTCGCG
56341	CAGACAAGGT	CATCACAGAC	ACCATGGTGG	CTCCGGCCCC	CGGGGACTCG	AAGGAGCCGC
56401	CCTCGAAGAT	CGCGGTCCCT	ATCGTCGCCA	TCGCCGGCTC	GGACGATGTG	ATCGTGCCTC
56461	CAAGCGACGT	TCAGGATCTA	CAATCTCGCA	CCACGGAGCG	CTTCTATATG	CATCTCCTTC
56521	CCGGAGATCA	CGAGTTTCTC	GTCGATCGAG	GGCGCGAGAT	CATGCACATC	GTCGACTCGC
56581	ATCTCAATCC	GCTGCTCGCC	GCGAGGACGA	CGTCGTCAGG	CCCCGCGTTC	GAGGCAAAAT

56641 GATGGCAGCC TCCCTCGGGC GCGCGAGATG GTTGGGAGCA GCGTGGGTGC TGGTGGCCGG
56701 CGGCAGGCAG CGGAGGCTCA TGAGCCTTCC TGGAAGTTTG CAGCATAGGA GATTTTATGA
56761 CACAGGAGCA AGCGAATCAG AGTGAGACGA AGCCTGCTTT CGACTTCAAG CCGTTCGCGC
56821 CTGGGTACGC GGAGGACCCG TTTCCCGCGA TCGAGCGCCT GAGAGAGGCA ACCCCCATCT
56881 TCTACTGGGA TGAAGGCCGC TCCTGGGTCC TCACCCGATA CCACGACGTG TCGGCGGTGT
56941 TCCGCGACGA ACGCTTCGCG GTCAGTCGAG AAGAATGGGA ATCGAGCGCG GAGTACTCGT
57001 CGGCCATTCC CGAGCTCAGC GATATGAAGA AGTACGGATT GTTCGGGCTG CCGCCGGAGG
57061 ATCACGCTCG GGTCCGCAAG CTCGTCAACC CATCGTTTAC GTCACGCGCG ATCGACCTGC
57121 TGCGCGCCGA AATACAGCGC ACCGTCGACC AGCTGCTCGA TGCTCGCTCC GGACAAGAGG
57181 AGTTCGACGT TGTGCGGGAT TACGCGGAGG GAATCCCGAT GCGTGCGATC AGCGCTCTGT
57241 TGAAGGTTCC GGCCGAGTGT GACGAGAAGT TCCGTCGCTT CGGCTCGGCG ACTGCGCGCG
57301 CGCTCGGCGT GGGTTTGGTG CCCCGGGTCG ATGAGGAGAC CAAGACCCTG GTCGCGTCCG
57361 TCACCGAGGG GCTCGCGCTG CTCCATGGCG TCCTCGATGA GCGGCGCAGG AACCCGCTCG
57421 AAAATGACGT CTTGACGATG CTGCTTCAGG CCGAGGCCGA CGGCAGCAGG CTGAGCACGA
57481 AGGAGCTGGT CGCGCTCGTG GGTGCGATTA TCGCTGCTGG CACCGATACC ACGATCTACC
57541 TTATCGCGTT CGCTGTGCTC AACCTGCTGC GGTCGCCCGA GGCGCTCGAG CTGGTGAAGG
57601 CCGAGCCCGG GCTCATGAGG AACGCGCTCG ATGAGGTGCT CCGCTTCGAC AATATCCTCA
57661 GAATAGGAAC TGTGCGTTTC GCCAGGCAGG ACCTGGAGTA CTGCGGGGCA TCGATCAAGA
57721 AAGGGGAGAT GGTCTTTCTC CTGATCCCGA GCGCCCTGAG AGATGGGACT GTATTCTCCA
57781 GGCCAGACGT GTTTGATGTG CGACGGGACA CGAGCGCGAG CCTCGCGTAC GGTAGAGGCC
57841 CCCATGTCTG CCCCGGGGTG TCCCTTGCTC GCCTCGAGGC GGAGATCGCC GTGGGCACCA
57901 TCTTCCGTAG GTTCCCCGAG ATGAAGCTGA AAGAAACTCC CGTGTTTGGA TACCACCCCG
57961 CGTTCCGGAA CATCGAATCA CTCAACGTCA TCTTGAAGCC CTCCAAAGCT GGATAACTCG
58021 CGGGGGCATC GCTTCCCGAA CCTCATTCTT TCATGATGCA ACTCGCGCGC GGGTGCTGTC
58081 TGCCGCGGGT GCGATTCGAT CCAGCGGACA AGCCCATTGT CAGCGCGCGA AGATCGAATC
58141 CACGGCCCGG AGAAGAGCCC GATGGCGAGC CCGTCCGGGT AACGTCGGAA GAAGTGCCGG
58201 GCGCCGCCCT GGGAGCGCAA AGCTCGCTCG CTCGCGCTCA GCGCGCCGCT TGCCATGTCC
58261 GGCCCTGCAC CCGCACCGAG GAGCCACCCG CCCTGATGCA CGGCCTCACC GAGCGGCAGG
58321 TTCTGCTCTC GCTCGTCGCC CTCGCGCTCG TCCTCCTGAC CGCGCGCGCC TTCGGCGAGC
58381 TCGCGCGGCG GCTGCGCCAG CCCGAGGTGC TCGGCGAGCT CTTCGGCGGC GTGGTGCTGG
58441 GCCCGTCCGT CGTCGGCGCG CTCGCTCCTG GGTTCCATCG AGTCCTCTTC CAGGATCCGG
58501 CGGTCGGGGG CGTGCTCTCC GGCATCTCCT GGATAGGCGC GCTCGTCCTG CTGCTCATGG
58561 CGGGTATCGA GGTCGATGTG AGCATTCTAC GCAAGGAGGC GCGCCCCGGG GCGCTCTCGG
58621 CGCTCGGCGC GATCGCGCCC CCGCTGCGCA CGCCGGGCCC GCTGGTGCAG CGCATGCAGG
58681 GCACGTTGAC GTGGGATCTC GACGTCTCGC CGCGACGCTC TGCGCAAGCC TGAGCCTCGG
58741 CGCCTGCTCG TACACCTCGC CGGTGCTCGC TCCGCCCGCG GACATCCGGC CGCCCCCCGC
58801 GGCCCAGCTC GAGCCGGACT CGCCGGATGA CGAGGCCGAC GAGGCGCTCC GCCCGTTCCG
58861 CGACGCGATC GCCGCGTACT CGGAGGCCGT TCGGTGGGCG GAGGCGGCGC AGCGGCCGCG
58921 GCTGGAGAGC CTCGTGCGGC TCGCGATCGT GCGGCTGGGC AAGGCGCTCG ACAAGGCACC
58981 TTTCGCGCAC ACGACGGCCG GCGTCTCCCA GATCGCCGGC AGACTTCCCC AGAAAACGAA
59041 TGCGGTCTGG TTCGATGTCG CCGCCCGGTA CGCGAGCTTC CGCGCGGCGA CGGAGCACGC
59101 GCTCCGCGAC GCGGCGTCGG CCACGGAGGC GCTCGCGGCC GGCCCGTACC GCGGATCGAG
59161 CAGCGTGTCC GCTGCCGTAG GGGAGTTTCG GGGGGAGGCG GCGCGCCTTC ACCCCGCGGA
59221 CCGCGTACCC GCGTCCGACC AGCAGATCCT GACCGCGCTG CGCGCAGCCG AGCGGGCGCT
59281 CATCGCGCTC TACACCGCGT TCGCCCGTGA GGAGTGAGCC TCTCTCGGGC GCAGCCGAGC
59341 GGCGGCGTGC CGGTTGTTCC CTCTTCGCAA CCATGACCGG AGCCGCGCCC GGTCCGCGCA
59401 GCGGCTAGCG CGCGTCGAGG CAGAGAGCGC TGGAGCGACA GGCGACGACC CGCCCGAGGG
59461 TGTCGAACGG ATTGCCGCAG CCCTCATTGC GGATCCCCTC CAGACACTCG TTCAGCGCCT
59521 TGGCGTCGAT GCCGCCTGGG CACTCGCCGA AGGTCAGCTC GTCGCGCCAG TCGGATCGGA
59581 TCTTGTTCGA GCACGCATCC TTGCTCGAAT ACTCCCGGTC TTGTCCGATG TTGTTGCACC
59641 GCGCCTCGCG GTCGCACCGC GCCGCCACGA TGCTATCGAC GGCGCTGCCG ACTGGCACCG
59701 GCGCCTCGCC TTGCGCGCCA CCCGGGGTTT GCGCCTCCCC GCCTGACCGC TTTTCGCCGC
59761 CGCACGCCGC CGCGAGCAGG CTCATTCCCG ACATCGAGAT CAGGCCCACG ACCAGTTTCC
59821 CAGCAATCTT TTGCATGGCT TCCCCTCCCT CACGACACGT CACATCAGAG ATTCTCCGCT

59881 CGGCTCGTCG GTTCGACAGC CGGCGACGGC CACGAGCAGA ACCGTCCCCG ACCAGAACAG
59941 CCGCATGCGG GTTTCTCGCA GCATGCCACG ACATCCTTGC GACTAGCGTG CCTCCGCTCG
60001 TGCCGAGATC GGCTGTCCTG TGCGACGGCA ATGTCCTGCG ATCGGCCGGG CAGGATCGAC
60061 CGACACGGGC GCCGGGCTGG AGGTGCCGCC ACGGGCTCGA AATGCGCTGT GGCAGGCGCC
60121 TCCATGCCCG CTGCCGGGAA CGCAGCGCCC GGCCAGCCTC GGGGCGACGC TGCGAACGGG
60181 AGATGCTCCC GGAGAGGCGC CGGGCACAGC CGAGCGCCGT CACCACCGTG CGCACTCGTG
60241 AGCGCTAGCT CCTCGGCATA GAAGAGACCG TCACTCCCGG TCCGTGTAGG CGATCGTGCT
60301 GATCAGCGCG TCCTCCGCCT GACGCGAGTC GAGCCGGGTA TGCTGCACGA CGATGGGCAC
60361 GTCCGATTCG ATCACGCTGG CATAGTCCGT ATCGCGCGGG ATCGGCTCGG GGTCGGTCAG
60421 ATCGTTGAAC CGGACGTGCC GGGTGCGCCT CGCTGGAACG GTCACCCGGT ACGGCCCGGC
60481 GGGGTCGCGG TCGCTGAAGT AGACGGTGAT GGCGACCTGC GCGTCCCGGT CCGACGCATT
60541 CAACAGGCAG GCCGTCTCAT GGCTCGTCAT CTGCGGCTCA GGTCCGTTGC TCCGGCCTGG
60601 GATGTAGCCC TCTGCGATTG CCCAGCGCGT CCGCCCGATC GGCTTGTCCA TGTGTCCTCC
60661 CTCCTGGCTC CTCTTTGGCA GCCTCCCTCT GCTGTCCAGG TGCGACGGCC TCTTCGCTCG
60721 ACGCGCTCGG GGCTCCATGG CTGAGAATCC TCGCCGAGCG CTCCTTGCCG ACCGGCGCGC
60781 TGAGCGCCGA CGGGCCTTGA AAGCACGCGA CCGGACACGG GATGCCGGCG CGACGAGGCC
60841 GCCCCGCGTC TGATCCCGAT CGTGGCATCA CGACGTCCGC CGACGCCTCG GCAGGCCGGC
60901 GTGAGCGCTG CGCGGTCATG GTCGTCCTCG CGTCACCGCC ACCCGCCGAT TCACATCCCA
60961 CCGCGGCACG ACGCTTGCTC AAACCGCGAC GACACGGCCG GGCGGCTGTG GTACCGGCCA
61021 GCCCGGACGC GAGGCCCGAG AGGGACAGTG GGTCCGCCGT GAAGCAGAGA GGCGATCGAG
61081 GTGGTGAGAT GAAACACGTT GACACGGGCC GACGAGTCGG CCGCCGGATA GGGCTCACGC
61141 TCGGTCTCCT CGCGAGCATG GCGCTCGCCG GCTGCGGCGG CCCGAGCGAG AAGACCGTGC
61201 AGGGCACGCG GCTCGCGCCC GGCGCCGATG CGCACGTCAC CGCCGACGTC GACGCCGACG
61261 CCGCGACCAC GCGGCTGGCG GTGGACGTCG TTCACCTCTC GCCGCCCGAG CGGATCGAGG
61321 CCGGCAGCGA GCGGTTCGTC GTCTGGCAGC GTCCGAACTC CGAGTCCCCG TGGCTACGGG
61381 TCGGAGTGCT CGACTACAAC GCTGCCAGCC GAAGAGGCAA GCTGGCCGAG ACGACCGTGC
61441 CGCATGCCAA CTTCGAGCTG CTCATCACCG TCGAGAAGCA GAGCAGCCCT CAGTCGCCAT
61501 CGTCTGCCGC CGTCATCGGG CCGACGTCCG TCGGGTAACA TCGCGCTATC AGCAGCGCTG
61561 AGCCCGCCAG CATGCCCCAG AGCCCTGCCT CGATCGCTTT CCCCATCATC CGTGCGCACT
61621 CCTCCAGCGA CGGCCGCGTC AAAGCAACCG CCGTGCCGGC GCGGCTCTAC GTGCGCGACA
61681 GGAGAGCGTC CTAGCGCGGC CTGCGCATCG CTGGAAGGAT CGGCGGAGCA TGGAGAAAGA
61741 ATCGAGGATC GCGATCTACG GCGCCGTCGC CGCCAACGTG GCGATCGCGG CGGTCAAGTT
61801 CATCGCCGCC GCCGTGACCG GCAGCTCTGC GATGCTCTCC GAGGGCGTGC ACTCCCTCGT
61861 CGATACCGCA GACGGGCTCC TCCTCCTGCT CGGCAAGCAC CGGAGCGCCC GCCCGCCCGA
61921 CGCCGAGCAT CCGTTCGGCC ACGGCAAGGA GCTCTATTTC TGGACGCTGA TCGTCGCCAT
61981 CATGATCTTC GCCGCGGGCG GCGGCGTCTC GATCTACGAA GGGATCTTGC ACCTCTTGCA
62041 CCCGCGCTCG ATCGAGGATC CGACGTGGAA CTACGTTGTC CTCGGCGCAG CGGCCGTCTT
62101 CGAGGGGACG TCGCTCGCCA TCTCGATCCA CGAGTTCAAG AAGAAAGACG GACAGGGCTA
62161 CGTCGCGGCG ATGCGGTCCA GCAAGGACCC GACGACGTTC ACGATCGTCC TGGAGGATTC
62221 CGCGGCGCTC GCCGGGCTCG CCATCGCCTT CCTCGGCGTC TGGCTTGGGC ACCGCCTGGG
62281 AAACCCCTAC CTCGACGGCG CGGCGTCGAT CGGCATCGGC CTCGTGCTCG CCGCGGTCGC
62341 GGTCTTCCTC GCCAGCCAGA GCCGTGGACT CCTCGTAGGG GAGAGCGCGG ACAGGGAGCT
62401 CCTCGCCGCG ATCCGCGCGC TCGCCAGCGC AGATCCTGGC GTGTCGGCGG TGGGGCGGCC
62461 CCTGACGATG CACTTCGGTC CGCACGAAGT CCTGGTCGTG CTGCGCATCG AGTTCGACGC
62521 CGCGCTCACG GCGTCCGGGG TCGCGGAGGC GATCGAGCGA ATCGAGACAC GGATACGGAG
62581 CGAGCGACCC GACGTGAAGC ACATCTACGT CGAGGCCAGG TCGCTCCACC AGCGCGCGAG
62641 GGCGTGACGC GCCGTGGAGA GACCGCTCGC GGCCTCCGCC ATCCTCCGCG GCGCCCGGGC
62701 TCGGGTAGCC CTCGCAGCAG GGCGCGCCTG GCGGGCAAAC CGTGAAGACG TCGTCCTTCG
62761 ACGCGAGGTA CGCTGGTTGC AAGTTGTCAC GCCGTATCGC GAGGTCCGGC AGCGCCGGAG
62821 CCCGGGCGGT CCGGGCGCAC GAAGGCCCGG CGAGCGCGGG CTTCGAGGGG GCGACGTCAT
62881 GAGGAAGGGC AGGGCGCATG GGGCGATGCT CGGCGGGCGA GAGGACGGCT GGCGTCGCGG
62941 CCTCCCCGGC GCCGGCGCGC TTCGCGCCGC GCTCCAGCGC GGTCGCTCGC GCGATCTCGC
63001 CCGGCGCCGG CTCATCGCCG CCGTGTCCCT CACCGGCGGC GCCAGCATGG CGGTCGTCTC
63061 GCTGTTCCAG CTCGGGATCA TCGAGCACCT GCCCGATCCT CCGCTTCCAG GGTTCGATTC

63121 GGCCAAGGTG ACGAGCTCCG ATATCGCGTT CGGGCTCACG ATGCCGGACG CGCCGCTCGC
63181 GCTCACCAGC TTCGCGTCCA ACCTGGCGCT GGCTGGCTGG GGAGGCGCCG AGCGCGCCAG
63241 GAACACCCCC TGGATCCCCG TCGCCGTGGC GGCCAAGGCG GCCGTCGAGG CGGCCGTGTC
63301 CGGATGGCTC CTCGTCCAGA TGCGACGGCG GGAGAGGGCC TGGTGCGCGT ACTGCCTGGT
63361 CGCCATGGCG GCCAACATGG CCGTGTTCGC GCTCTCGCTC CCGGAAGGGT GGGCGGCGCT
63421 GAGGAAGGCG CGAGCGCGCT CGTGACAGGG CCGTGCGGGC GCCGCGGCCA TCGGAGGCCG
63481 GCGTGCACCC GCTCCGTCAC GCCCCGGCCC GCGCCGCGGT GAGCTGCCGC GGACAGGGCG
63541 CGTACCGTGG ACCCCGCACG CGCCGCGTCG ACGGACATCC CCGGCGGCTC GCGCGGCGCG
63601 GCCGGCGCAA CTCCGGCCCG CCGCCGGGCA TCGACATCTC CCGCGAGCAA GGGCACTCCG
63661 CTCCTGCCCG CGTCCGCGAA CGATGGCTGC GCTGTTTCCA CCCTGGAGCA ACTCCGTTTA
63721 CCGCGTGGCG CTCGTCGGGC TCATCGCCTC GGCGGGCGGC GCCATCCTCG CGCTCATGAT
63781 CTACGTCCGC ACGCCGTGGA AGCGATACCA GTTCGAGCCC GTCGATCAGC CGGTGCAGTT
63841 CGATCACCGC CATCACGTGC AGGACGATGG CATCGATTGC GTCTACTGCC ACACCACGGT
63901 GACCCGCTCG CCGACGGCGG GGATGCCGCC GACGGCCACG TGCATGGGGT GCCACAGCCA
63961 GATCTGGAAT CAGAGCGTCA TGCTCGAGCC CGTGCGGCGG AGCTGGTTCT CCGGCATGCC
64021 GATCCCGTGG AACCGGGTGA ACTCCGTGCC CGACTTCGTT TATTTCAACC ACGCGATTCA
64081 CGTGAACAAG GGCGTGGGCT GCGTGAGCTG CCACGGGCGC GTGGACGAGA TGGCGGCCGT
64141 CTACAAGGTG GCGCCGATGA CGATGGGCTG GTGCCTGGAG TGCCATCGCC TGCCGGAGCC
64201 GCACCTGCGC CCGCTCTCCG CGATCACCGA CATGCGCTGG GACCCGGGGG AACGGAGGGA
64261 CGAGCTCGGG GCGAAGCTCG CGAAGGAGTA CGGGGTCCGG CGGCTCACGC ACTGCACAGC
64321 GTGCCATCGA TGAACGATGA ACAGGGGATC TCCGTGAAAG ACGCAGATGA GATGAAGGAA
64381 TGGTGGCTAG AAGCGCTCGG GCCGGCGGGA GAGCGCGCGT CCTACAGGCT GCTGGCGCCG
64441 CTCATCGAGA GCCCGGAGCT CCGCGCGCTC GCCGCGGGCG AACCGCCCCG GGGCGTGGAC
64501 GAGCCGGCGG GCGTCAGCCG CCGCGCGCTG CTCAAGCTGC TCGGCGCGAG CATGGCGCTC
64561 GCCGGCGTCG CGGGCTGCAC CCCGCATGAG CCCGAGAAGA TCCTGCCGTA CAACGAGACC
64621 CCGCCCGGCG TCGTGCCGGG TCTCTCCCAG TCCTACGCGA CGAGCATGGT GCTCGACGGG
64681 TATGCCATGG GCCTCCTCGC CAAGAGCTAC GCGGGGCGGC CCATCAAGAT CGAGGGCAAC
64741 CCCGCGCACC CGGCGAGCCT CGGCGCGACC GGCGTCCACG AGCAGGCCTC GATCCTCTCG
64801 CTGTACGACC CGTACCGCGC GCGCGCGCCG ACGCGCGGCG GCCAGGTCGC GTCGTGGGAG
64861 GCGCTCTCCG CGCGCTTCGG CGGCGACCGC GAGGACGGCG GCGCTGGCCT CCGCTTCGTC
64921 CTCCAGCCCA CGAGCTCGCC CCTCATCGCC GCGCTGATCG AGCGCGTCCG GCGCAGGTTC
64981 CCCGGCGCGC GGTTCACCTT CTGGTCGCCG GTCCACGCCG AGCAAGCGCT CGAAGGCGCG
65041 CGGGCGGCGC TCGGCCTCAG GCTCTTGCCT CAGCTCGACT TCGACCAGGC CGAGGTGATC
65101 CTCGCCCTGG ACGCGGACTT CCTCGCGGAC ATGCCGTTCA GCGTGCGCTA TGCGCGCGAC
65161 TTCGCCGCGC GCCGCCGACC CGCGAGCCCG GCGGCGGCCA TGAACCGCCT CTACGTCGCG
65221 GAGGCGATGT TCACGCCCAC GGGGACGCTC GCCGACCACC GGCTCCGCGT GCGGCCCGCC
65281 GAGGTCGCGC GCGTCGCGGC CGGCGTCGCG GCGGAGCTCG TGCACGGCCT CGGCCTGCGC
65341 CCGCGCGGGA TCACGGACGC CGACGCCGCC GCGCTGCGCG CGCTCCGCCC CCCGGACGGC
65401 GAGGGGCACG GCGCCTTCGT CCGGGCGCTC GCGCGCGATC TCGCGCGCGC GGGGGGCGCC
65461 GGCGTCGCCG TCGTCGGCGA CGGCCAGCCG CCCATCGTCC ACGCCCTCGG GCACGTCATC
65521 AACGCCGCGC TCCGCAGCCG GGCGGCCTGG ATGGTCGATC CTGTGCTGAT CGACGCGGGC
65581 CCCTCCACGC AGGGCTTCTC CGAGCTCGTC GGCGAGCTCG GGCGCGGCGC GGTCGACACC
65641 TGATCCTCCT CGACGTGAAC CCCGTGTACG CCGCGCCGGC CGACGTCGAT TTCGCGGGCC
65701 TCCTCGCGCG CGTGCCCACG AGCTTGAAGG CCGGGCTCTA CGACGACGAG ACCGCCCGCG
65761 CTTGCACGTG GTTCGTGCCG ACCCGGCATT ACCTCGAGTC GTGGGGGGAC GCGCGGGCGT
65821 ACGACGGGAC GGTCTCGTTC GTGCAACCCC TCGTCCGGCC GCTGTTCGAC GGCCGGGCGG
65881 TGCCCGAGCT GCTCGCCGTC TTCGCGGGGG ACGAGCGCCC GGATCCCCGG CTGCTGCTGC
65941 GCGAGCACTG GCGCGGCGCG CGCGGAGAGG CGGATTTCGA GGCCTTCTGG GGCGAGGCAT
66001 TGAAGCGCGG CTTCCTCCCT GACAGCGCCC GGCCGAGGCA GACACCGGAT CTCGCGCCGG
66061 CCGACCTCGC CAAGGAGCTC GCGCGGCTCG CCGCCGCGCC GCGGCCGGCC GGCGGCGCGC
66121 TCGACGTGGC GTTCCTCAGG TCGCCGTCGG TCCACGACGG CAGGTTCGCC AACAACCCCT
66181 GGCTGCAAGA GCTCCCGCGG CCGATCACCA GGCTCACCTG GGGCAACGCC GCCATGATGA
66241 GCGCGGCGAC CGCGGCGCGG CTCGGCGTCG AGCGCGGCGA TGTCGTCGAG CTCGCGCTGC
66301 GCGGCCGTAC GATCGAGATC CCGGCCGTCG TCGTCCGCGG GCACGCCGAC GACGTGATCA

```
66361 GCGTCGACCT CGGCTACGGG CGCGACGCCG GCGAGGAGGT CGCGCGCGGG GTGGGCGTGT
66421 CGGCGTATCG GATCCGCCCG TCCGACGCGC GGTGGTTCGC GGGGGGCCTC TCCGTGAGGA
66481 AGACCGGCGC CACGGCCGCG CTCGCGCTGG CTCAGATCGA GCTGTCCCAG CACGACCGTC
66541 CCATCGCGCT CCGGAGGACG CTGCCGCAGT ACCGTGAACA GCCCGGTTTC GCGGAGGAGC
66601 ACAAGGGGCC GGTCCGCTCG ATCCTGCCGG AGGTCGAGTA CACCGGCGCG CAATGGGCGA
66661 TGTCCATCGA CATGTCGATC TGCACCGGGT GCTCCTCGTG CGTCGTGGCC TGTCAGGCCG
66721 AGAACAACGT CCTCGTCGTC GGCAAGGAGG AGGTGATGCA CGGCCGCGAG ATGCAGTGGT
66781 TGCGGATCGA TCAGTACTTC GAGGGTGGAG GCGACGAGGT GAGCGTCGTC AACCAGCCGA
66841 TGCTCTGCCA GCACTGCGAG AAGGCGCCGT GCGAGTACGT CTGTCCGGTG AACGCGACGG
66901 TCCACAGCCC CGATGGCCTC AACGAGATGA TCTACAACCG ATGCATCGGG ACGCGCTTTT
66961 GCTCCAACAA CTGTCCGTAC AAGATCCGGC GGTTCAATTT CTTCGACTAC AATGCCCACG
67021 TCCCGTACAA CGCCGGCCTC CGCAGGCTCC AGCGCAACCC GGACGTCACC GTCCGCGCCC
67081 GCGGCGTCAT GGAGAAATGC ACGTACTGCG TGCAGCGGAT CCGAGAGGCG GACATCCGCG
67141 CGCAGATCGA GCGGCGGCCG CTCCGGCCGG GCGAGGTGGT CACCGCCTGC CAGCAGGCCT
67201 GTCCGACCGG CGCGATCCAG TTCGGGTCGC TGGATCACGC GGATACAAAG ATGGTCGCGT
67261 GGCGCAGGGA GCCGCGCGCG TACGCCGTGC TCCACGACCT CGGCACCCGG CCGCGGACGG
67321 AGTACCTCGC CAAGATCGAG AACCCGAACC CGGGGCTCGG GGCGGAGGGC GCCGAGAGGC
67381 GACCCGGAGC CCCGAGCGTC AAACCCGCGC TCGGGGCGGA GGGCGCCGAG AGGCGACCCG
67441 GAGCCCCGAG CGTCAAACCG GAGATTGAAT GAGCCATGGC GGGCCCGCTC ATCCTGGACG
67501 CACCGACCGA CGATCAGCTG TCGAAGCAGC TCCTCGAGCC GGTATGGAAG CCGCGCTCCC
67561 GGCTCGGCTG GATGCTCGCG TTCGGGCTCG CGCTCGGCGG CACGGGCCTG CTCTTCCTCG
67621 CGATCACCTA CACCGTCCTC ACCGGGATCG GCGTGTGGGG CAACAACATC CCGGTCGCCT
67681 GGGCCTTCGC GATCACCAAC TTCGTCTGGT GGATCGGGAT CGGCCACGCC GGGACGTTCA
67741 TCTCCGCGAT CCTCCTCCTG CTCGAGCAGA AGTGGCGGAC GAGCATCAAC CGCTTCGCCG
67801 AGGCGATGAC GCTCTTCGCG GTCGTCCAGG CCGGCCTCTT TCCGGTCCTC CACCTCGGCC
67861 GCCCCTGGTT CGCCTACTGG ATCTTCCCGT ACCCCGCGAC GATGCAGGTG TGGCCGCAGT
67921 TCCGGAGCGC GCTGCCGTGG GACGCCGCCG CGATCGCGAC CTACTTCACG GTGTCGCTCC
67981 TGTTCTGGTA CATGGGCCTC GTCCCGGATC TGGCGGCGCT GCGCGACCAC GCCCCGGGCC
68041 GCGTCCGGCG GGTGATCTAC GGGCTCATGT CGTTCGGCTG GCACGGCGCG GCCGACCACT
68101 TCCGGCATTA CCGGGTGCTG TACGGGCTGC TCGCGGGGCT CGCGACGCCC CTCGTCGTCT
68161 CGGTGCACTC GATCGTGAGC AGCGATTTCG CGATCGCCCT GGTGCCCGGC TGGCACTCGA
68221 CGCTCTTTCC GCCGTTCTTC GTCGCGGGCG CGATCTTCTC CGGGTTCGCG ATGGTGCTCA
68281 CGCTGCTCAT CCCGGTGCGG CGGATCTACG GGCTCCATAA CGTCGTGACC GCGCGCCACC
68341 TCGACGATCT CGCGAAGATG ACGCTCGTGA CCGGCTGGAT CGTCATCCTC TCGTACATCA
68401 TCGAGAACTT CCTCGCCTGG TACAGCGGCT CGGCGTACGA GATGCATCAG TTTTTCCAGA
68461 CGCGCCTGCA CGGCCCGAAC AGCGCCGCCT ACTGGGCCCA GCACGTCTGC AACGTGCTCG
68521 TCATCCAGCT CCTCTGGAGC GAGCGGATCC GGACGAGCCC CGTCGCGCTC TGGCTCATCT
68581 CCCTCCTGGT CAACGTCGGG ATGTGGAGCG AGCGGTTCAC GCTCATCGTG ATGTCGCTCG
68641 AGCAAGAGTT CCTCCCGTCC AAGTGGCACG GCTACAGCCC GACGTGGGTG GACTGGAGCC
68701 TCTTCATCGG GTCAGGCGGC TTCTTCATGC TCCTGTTCCT GAGCTTTTTG CGCGTCTTTC
68761 CGTTCATCCC CGTCGCGGAG GTCAAGGAGC TCAACCATGA AGAGCTGGAG AAGGCTCGGG
68821 GCGAGGGGGG CCGCTGATGG AGACCGGAAT GCTCGGCGAG TTCGATGACC CGGAGGCGAT
68881 GCTCCATGCG ATCCGAGAGC TCAGGCGGCG CGGCTACCGC CGGGTGGAAG CGTTCACGCC
68941 CTATCCGGTG AAGGGGCTCG ACGAGGCGCT CGGCCTCCCG CGCTCGAACC TCAACCGGAT
69001 GGTGCTGCCC TTCGCGATCC TGGGGGTCGT GGGCGGCTAC TTCGTCCAGT GGTTCTGCAA
69061 CGCTTTCCAC TATCCGCTGA ACGTGGGCGG GCGCCCGCTG AACTCGGCGC CGGCGTTCAT
69121 CCCGATCACG TTCGAGATGG GGGTGCTCTC CACCTCGATC TTCGGCGTGC TCATCGGCTT
69181 TTACCTGACG AGGCTGCCGA GGCTCTACCT CCCGCTCTTC GACGCCCCGG GCTTCGAGCG
69241 CGTCACGCTG GATCGGTTTC TGGTCGGGCT CGACGACACG GAACCTTCCT TCTCGAGCGC
69301 CCAGGCGGAG CGCGACCTCC TCGCGCTCGG CGCCCGGCGC GTCGTCGTCG CGAGGAGGCG
69361 CGAGGAGCCA TGAGGGCCGG CGCCCCGGCT CGCCCTCTCG GGCGCGCGCT CGCGCCGTTC
69421 GCCCTCGTCC TGCTCGCCGG GTGCCGCGAG AAGGTGCTGC CCGAGCCGGA CTTCGAGCGG
69481 ATGATCCGCC AGGAGAAATA CGGACTCTGG GAGCCGTGCG AGCACTTCGA CGACGGCCGC
69541 GCGATGCAGC ACCCGCCCGA GGGGACCGTC GCGCGCGGGC GCGTCACCGG GCCGCCCGGC
```

```
69601 TATCTCCAGG GCGTCCTCGA CGGGGCGTAC GTCACGGAGG TGCCGCTCTT GCTCACGGTC
69661 GAGCTCGTGC AGCGCGGCCG GCAGCGCTTC GAGACCTTCT GCGCGCCGTG CCACGGGATC
69721 CTCGGCGACG GCAGCTCGCG CGTGGCGACG AACATGACGC TGCGCCCGCC CCCGTCGCTC
69781 ATCGGACCCG AGGCGCGGAG CTTCCCGCCG GGCAGGATCT ACCAGGTCAT CATCGAGGGC
69841 TACGGCCTGA TGCCGCGCTA CTCGGACGAT CTGCCCGACA TCGAAGAGCG CTGGGCCGTG
69901 GTCGCCTACG TGAAGGCGCT TCAGCTGAGC CGCGGAGTGG CCGCGGGCGC CCTCCCGCCA
69961 GCGCTCCGCG GCCGGGCAGA GCAGGAGCTG CGATGAACAG GGATGCCATC GAGTACAAGG
70021 GCGGCGCGAC GATCGCGGCC TCGCTCGCGA TCGCGGCGCT CGGCGCGGTC GCCGCGATCG
70081 TCGGCGGCTT CGTCGATCTC CGCCGGTTCT TCTTCTCGTA CCTCGCCGCG TGGTCGTTCG
70141 CGGTGTTTCT GTCCGTGGGC GCGCTCGTCA CGCTCCTCAC CTGCAACGCC ATGCGCGCGG
70201 GCTGGCCCAC GGCGGTGCGC CGCCTCCTCG AGACGATGGT GGCGCCGCTG CCTCTGCTCG
70261 CGGCGCTCTC CGCGCCGATC CTGGTCGGCC TGGACACGCT GTATCCGTGG ATGCACCCCG
70321 AGCGGATCGC CGGCGAGCAC GCGCGGCGCA TCCTCGAGCA CAGGGCGCCC TACTTCAATC
70381 CAGGCTTCTT CGTCGTGCGC TCGGCGATCT ACTTCGCGAT CTGGATCGCC GTCGCCCTCG
70441 TGCTCCGCCG GCGATCGTTC GCGCAGGACC GTGAGCCGAG GGCCGACGTC AAGGACGCGA
70501 TGTATGGCCT GAGCGGCGCC ATGCTGCCGG TCGTGGCGAT CACGATCGTC TTCTCGTCGT
70561 TCGACTGGCT CATGTCCCTC GACGCGACCT GGTACTCGAC GATGTTCCCG GTCTACGTGT
70621 TCGCGAGCGC CTTCGTGACC GCCGTCGGCG CGCTCACGGT CCTCTCGTAT GCCGCGCAGA
70681 CGTCCGGCTA CCTCGCGAGG CTGAACGACT CGCACTATTA CGCGCTCGGG CGGCTGCTCC
70741 TCGCGTTCAC GATATTCTGG GCCTATGCGG CCTATTTCCA GTTCATGTTG ATCTGGATCG
70801 CGAACAAGCC CGATGAGGTC GCCTTCTTCC TCGACCGCTG GGAAGGGCCC TGGCGGCCGA
70861 CCTCCGTGCT CGTCGTCCTC ACGCGGTTCG TCGTCCCGTT CCTGATCCTG ATGTCGTACG
70921 CGATCAAGCG GCGCCCGCGC CAGCTCTCGT GGATGGCGCT CTGGGTCGTC GTCTCCGGCT
70981 ACATCGACTT TCACTGGCTC GTGGTGCCGG CGACAGGGCG CCACGGGTTC GCCTATCACT
71041 GGCTCGACCT CGCGACCCTG TGCGTCGTGG GCGGCCTCTC GACCGCGTTC GCCGCGTGGC
71101 GGCTGCGAGG GCGGCCGGTG GTCCCGGTCC ACGACCCGCG GCTCGAAGAG GCCTTTGCGT
71161 ACCGGAGCAT ATGATGTTCC GTTTCCGTCA CAGCGAGGTT CGCCAGGAGG AGGACACGCT
71221 CCCCTGGGGG CGCGTGATCC TCGCGTTCGC CGTCGTGCTC GCGATCGGCG GCGCGCTGAC
71281 GCTCTGGGCC TGGCTCGCGA TGCGGGCCCG CGAGGCGGAT CTGCGGCCCT CCCTCGCGTT
71341 CCCCGAGAAG GATCTCGGGC CGCGGCGCGA GGTCGGCATG GTCCAGCAGT CGCTGTTCGA
71401 CGAGGCGCGC CTGGGCCAGC AGCTCGTCGA CGCGCAGCGC GCGGAGCTCC GCCGCTTCGG
71461 CGTCGTCGAT CGGGAGAGGG GCATCGTGAG CATCCCGATC GACGACGCGA TCGAGCTCAT
71521 GGTGGCGGGG GGCGCGCGAT GAGCCGGGCC GTCGCCGTGG CCCTCCTGCT GGCAGCCGGC
71581 CTCGTGTCGC GCCCGGGCGC CGCGTCCGAG CCCGAGCGCG CGCGCCCCGC GCTGGGCCCG
71641 TCCGCGGCCG ACGCCGCGCC GGCGAGCGAC GGCTCCGGCG CGGAGGAGCC GCCCGAAGGC
71701 GCCTTCCTGG AGCCCACGCG CGGGGTGGAC ATCGAGGAGC GCCTCGGCCG CCCGGTGGAC
71761 CGCGAGCTCG CCTTCACCGA CATGGACGGG CGGCGGGTGC GCCTCGGCGA CTACTTCGCC
71821 GACGGCAAGC CCCTCCTCCT CGTCCTCGCG TACTACCGGT GTCCCGCGCT GTGCGGCCTC
71881 GTGCTGCGCG GCGCCGTCGA GGGGCTGAAG CTCCTCCCGT ACCGGCTCGG CGAGCAGTTC
71941 CACGCGCTCA CGGTCAGCTT CGACCCGCGC GAGCGCCCGG CGGCCGCDD
```

Example 2

Construction of a *Myxococcus xanthus* Expression Vector

The DNA providing the integration and attachment function of phage Mx8 was inserted into commercially available pACYC184 (New England Biolabs). An ~2360 bp MfeI-SmaI from plasmid pPLH343, described in Salmi *et al.*, Feb. 1998, J. Bact. 180(3): 614-621, was isolated and ligated to the large EcoRI-XmnI restriction fragment of

plasmid pACYC184. The circular DNA thus formed was ~6 kb in size and called plasmid pKOS35-77.

Plasmid pKOS35-77 serves as a convenient plasmid for expressing recombinant PKS genes of the invention under the control of the epothilone PKS gene promoter. In one illustrative embodiment, the entire epothilone PKS gene with its homologous promoter is inserted in one or more fragments into the plasmid to yield an expression vector of the invention.

Sub B7

The present invention also provides expression vectors in which the recombinant PKS genes of the invention are under the control of a *Myxococcus xanthus* promoter. To construct an illustrative vector, the promoter of the pilA gene of *M. xanthus* was isolated as a PCR amplification product. Plasmid pSWU357, which comprises the pilA gene promoter and is described in Wu and Kaiser, Dec. 1997, J. Bact. 179(24):7748-7758, was mixed with PCR primers Seq1 and Mxpil1 primers:

Seq1: 5'-AGCGGATAACAATTTCACACAGGAAACAGC-3'; and

Mxpil1: 5'-TTAATTAAGAGAAGGTTGCAACGGGGGGC-3',

and amplified using standard PCR conditions to yield an ~800 bp fragment. This fragment was cleaved with restriction enzyme KpnI and ligated to the large KpnI-EcoRV restriction fragment of commercially available plasmid pLitmus 28 (New England Biolabs). The resulting circular DNA was designated plasmid pKOS35-71B.

The promoter of the pilA gene from plasmid pKOS35-71B was isolated as an ~800 bp EcoRV-SnaBI restriction fragment and ligated with the large MscI restriction fragment of plasmid pKOS35-77 to yield a circular DNA ~6.8 kb in size. Because the ~800 bp fragment could be inserted in either one of two orientations, the ligation produced two plasmids of the same size, which were designated as plasmids pKOS35-82.1 and pKOS35-82.2. Restriction site and function maps of these plasmids are presented in Figure 3.

Plasmids pKOS35-82.1 and pKOS35-82.2 serve as convenient starting materials for the vectors of the invention in which a recombinant PKS gene is placed under the control of the *Myxococcus xanthus pilA* gene promoter. These plasmids comprise a single PacI restriction enzyme recognition sequence placed immediately downstream of the

transcription start site of the promoter. In one illustrative embodiment, the entire epothilone PKS gene without its homologous promoter is inserted in one or more fragments into the plasmids at the PacI site to yield expression vectors of the invention.

Sub B8

The sequence of the pilA promoter in these plasmids is shown below.

```
CGACGCAGGTGAAGCTGCTTCGTGTGCTCCAGGAGCGGAAGGTGAAGCCGGTCGGCAGCGCCGCGGAGATT
CCCTTCCAGGCGCGTGTCATCGCGGCAACGAACCGGCGGCTCGAAGCCGAAGTAAAGGCCGGACGCTTTCG
TGAGGACCTCTTCTACCGGCTCAACGTCATCACGTTGGAGCTGCCTCCACTGCGCGAGCGTTCCGGCGACG
TGTCGTTGCTGGCGAACTACTTCCTGTCCAGACTGTCGGAGGAGTTGGGGCGACCCGGTCTGCGTTTCTCC
CCCGAGACACTGGGGCTATTGGAGCGCTATCCCTTCCCAGGCAACGTGCGGCAGCTGCAGAACATGGTGGA
GCGGGCCGCGACCCTGTCGGATTCAGACCTCCTGGGGCCCTCCACGCTTCCACCCGCAGTGCGGGGCGATA
CAGACCCCGCCGTGCGTCCCGTGGAGGGCAGTGAGCCAGGGCTGGTGGCGGGCTTCAACCTGGAGCGGCAT
CTCGACGACAGCGAGCGGCGCTATCTCGTCGCGGCGATGAAGCAGGCCGGGGGCGTGAAGACCCGTGCTGC
GGAGTTGCTGGGCCTTTCGTTCCGTTCATTCCGCTACCGGTTGGCCAAGCATGGGCTGACGGATGACTTGG
AGCCCGGGAGCGCTTCGGATGCGTAGGCTGATCGACAGTTATCGTCAGCGTCACTGCCGAATTTTGTCAGC
CCTGGACCCATCCTCGCCGAGGGGATTGTTCCAAGCCTTGAGAATTGGGGGGCTTGGAGTGCGCACCTGGG
TTGGCATGCGTAGTGCTAATCCCATCCGCGGGCGCAGTGCCCCCCGTTGCAACCTTCTCTTAATTAA
```

To make the recombinant *Myxococcus xanthus* host cells of the invention, *M. xanthus* cells are grown in CYE media (Campos and Zusman, 1975, Regulation of development in *Myxococcus xanthus*: effect of 3': 5'-cyclic AMP, ADP, and nutrition, Proc. Natl. Acad. Sci. USA 72: 518-522) to a Klett of 100 at 30°C at 300 rpm. The remainder of the protocol is conducted at 25°C unless otherwise indicated. The cells are then pelleted by centrifugation (8000 rpm for 10 min. in an SS34 or SA600 rotor) and resuspended in deionized water. The cells are again pelleted and resuspended in 1/100th of the original volume.

DNA (one to two µL) is electroporated into the cells in a 0.1 cm cuvette at room temperature at 400 ohm, 25 µFD, 0.65 V with a time constant in the range of 8.8 - 9.4. The DNA should be free of salts and so should be resuspended in distilled and deionized water or dialyzed on a 0.025 µm Type VS membrane (Millipore). For low efficiency electroporations, spot dialyze the DNA, and allow outgrowth in CYE. Immediately after electroporation, add 1 mL of CYE, and pool the cells in the cuvette with an additional 1.5 mL of CYE previously added to a 50 mL Erlenmeyer flask (total volume 2.5 ml). Allow the cells to grow for four to eight hours (or overnight) at 30 to 32°C at 300 rpm to allow for expression of the selectable marker. Then, plate the cells in CYE soft agar on plates with selection. If kanamycin is the selectable marker, then typical yields are 10^3 to 10^5

per µg of DNA. If streptomycin is the selectable marker, then it must be included in the top agar, because it binds agar.

With this procedure, the recombinant DNA expression vectors of the invention are electroporated into *Myxococcus* host cells that express recombinant PKSs of the invention and produce the epothilone, epothilone derivatives, and other novel polyketides encoded thereby.

Example 3

Construction of a Bacterial Artificial Chromosome (BAC) for Expression of Epothilone in *Myxococcus xanthus*

To express the epothilone PKS and modification enzyme genes in a heterologous host to produce epothilones by fermentation, *Myxococcus xanthus*, which is closely related to *Sorangium cellulosum* and for which a number of cloning vectors are available, can also be employed in accordance with the methods of the invention. Because both *M. xanthus* and *S. cellulosum* are myxobacteria, it is expected that they share common elements of gene expression, translational control, and post translational modification (if any), thereby enhancing the likelihood that the epo genes from *S. cellulosum* can be expressed to produce epothilone in *M. xanthus*. Secondly, *M. xanthus* has been developed for gene cloning and expression. DNA can be introduced by electroporation, and a number of vectors and genetic markers are available for the introduction of foreign DNA, including those that permit its stable insertion into the chromosome. Finally, *M. xanthus* can be grown with relative ease in complex media in fermentors and can be subjected to manipulations to increase gene expression, if required.

To introduce the epothilone gene cluster into *Myxococcus xanthus*, one can build the epothilone cluster into the chromosome by using cosmids of the invention and homologous recombination to assemble the complete gene cluster. Alternatively, the complete epothilone gene cluster can be cloned on a bacterial artificial chromosome (BAC) and then moved into *M. xanthus* for integration into the chromosome.

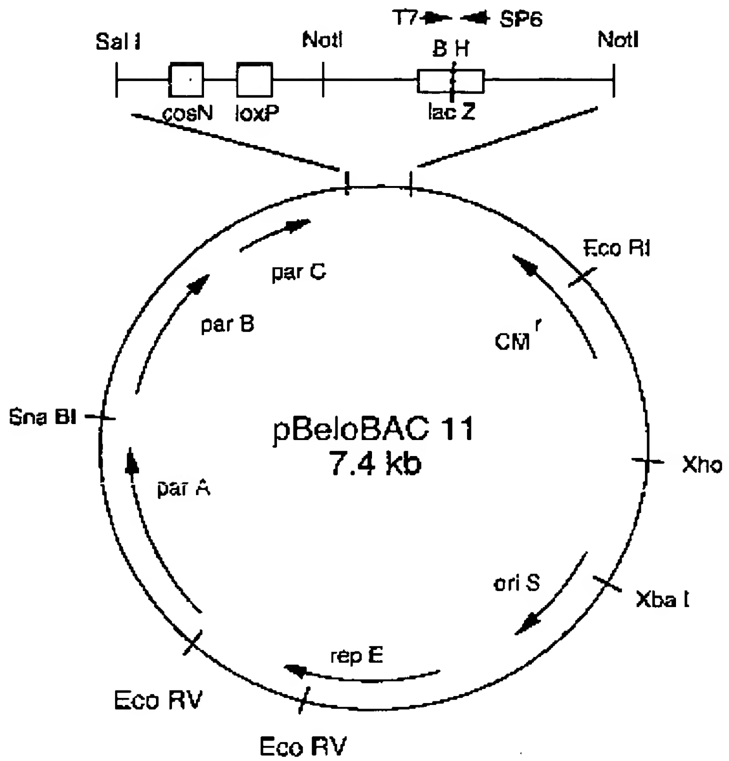
To assemble the gene cluster from cosmids pKOS35-70.1A2, and pKOS35-79.85, small regions of homology from these cosmids have to be introduced into *Myxococcus*

xanthus to provide recombination sites for larger pieces of the gene cluster. As shown in Figure 4, plasmids pKOS35-154 and pKOS90-22 are created to introduce these recombination sites. The strategy for assembling the epothilone gene cluster in the *M. xanthus* chromosome is shown in Figure 5. Initially, a neutral site in the bacterial chromosome is chosen that does not disrupt any genes or transcriptional units. One such region is downstream of the devS gene, which has been shown not to affect the growth or development of *M. xanthus*. The first plasmid, pKOS35-154, is linearized with DraI and electroporated into *M. xanthus*. This plasmid contains two regions of the dev locus flanking two fragments of the epothilone gene cluster. Inserted in between the epo gene regions are the kanamycin resistance marker and the galK gene. Kanamycin resistance arises in colonies if the DNA recombines into the dev region by a double recombination using the dev sequence as regions of homology. This strain, K35-159, contains small regions of the epothilone gene cluster that will allow for recombination of pKOS35-79.85. Because the resistance markers on pKOS35-79.85 are the same as that for K35-159, a tetracycline transposon was transposed into the cosmid, and cosmids that contain the transposon inserted into the kanamycin marker were selected. This cosmid, pKOS90-23, was electroporated into K35-159, and oxytetracycline resistant colonies were selected to create strain K35-174. To remove the unwanted regions from the cosmid and leave only the epothilone genes, cells were plated on CYE plates containing 1% galactose. The presence of the galK gene makes the cells sensitive to 1% galactose. Galactose resistant colonies of K35-174 represent cells that have lost the galK marker by recombination or by a mutation in the galK gene. If the recombination event occurs, then the galactose resistant strain is sensitive to kanamycin and oxytetracycline. Strains sensitive to both antibiotics are verified by Southern blot analysis. The correct strain is identified and designated K35-175 and contains the epothilone gene cluster from module 7 through two open reading frames past the *epoL* gene.

To introduce modules 1 through module 7, the above process is repeated once more. The plasmid pKOS90-22 is linearized with DraI and electroporated into K35-175 to create K35-180. This strain is electroporated with the tetracycline resistant version of pKOS35-70.1A2, pKOS90-38, and colonies resistant to oxytetracycline are selected. This

creates strain K35-185. Recombinants that now have the whole epothilone gene cluster are selected by resistance to 1% galactose. This results in strain K35-188. This strain contains all the epothilone genes as well as all potential promoters. This strain is fermented and tested for the production of epothilones A and B.

To clone the whole gene cluster as one fragment, a bacterial artificial chromosome (BAC) library is constructed. First, SMP44 cells are embedded in agarose and lysed according to the BIO-RAD genomic DNA plug kit. DNA plugs are partially digested with restriction enzyme, such as Sau3AI or HindIII, and electrophoresed on a FIGE or CHEF gel. DNA fragments are isolated by electroeluting the DNA from the agarose or using gelase to degrade the agarose. The method of choice to isolate the fragments is electroelution, as described in Strong *et al.*, 1997, Nucleic Acids Res. 19: 3959-3961, incorporated herein by reference. The DNA is ligated into the BAC (pBeloBACII) cleaved with the appropriate enzyme. A map of pBeloBACII is shown below.



The DNA is electroporated into DH10B cells by the method of Sheng *et al.*, 1995, Nucleic Acids Res. 23: 1990-1996, incorporated herein by reference, to create an *S. cellulosum* genomic library. Colonies are screened using a probe from the NRPS region of the epothilone cluster. Positive clones are picked and DNA is isolated for restriction analysis to confirm the presence of the complete gene cluster. This positive clone is designated pKOS35-178.

To create a strain that can be used to introduce pKOS35-178, a plasmid, pKOS35-164, is constructed that contains regions of homology that are upstream and downstream of the epothilone gene cluster flanked by the dev locus and containing the kanamycin resistance galK cassette, analogous to plasmids pKOS90-22 and pKOS35-154. This plasmid is linearized with DraI and electroporated into *M. xanthus*, in accordance with the method of Kafeshi *et al.*, 1995, Mol. Microbiol. 15: 483-494, to create K35-183. The plasmid pKOS35-178 can be introduced into K35-183 by electroporation or by

transduction with bacteriophage P1 and chloramphenicol resistant colonies are selected. Alternatively, a version of pKOS35-178 that contains the origin of conjugative transfer from pRP4 can be constructed for transfer of DNA from *E. coli* to K35-183. This plasmid is made by first constructing a transposon containing the oriT region from RP4 and the tetracycline resistance maker from pACYC184 and then transposing the transposon *in vitro* or *in vivo* onto pKOS35-178. This plasmid is transformed into S17-1 and conjugated into *M. xanthus*. This strain, K35-190, is grown in the presence of 1% galactose to select for the second recombination event. This strain contains all the epothilone genes as well as all potential promoters. This strain will be fermented and tested for the production of epothilones A and B.

Besides integrating pKOS35-178 into the dev locus, it can also be integrated into a phage attachment site using integration functions from myxophages Mx8 or Mx9. A transposon is constructed that contains the integration genes and att site from either Mx8 or Mx9 along with the tetracycline gene from pACYC184. Alternative versions of this transposon may have only the attachment site. In this version, the integration genes are then supplied in trans by coelectroporation of a plasmid containing the integrase gene or having the integrase protein expressed in the electroporated strain from any constitutive promoter, such as the mgl promoter (see Magrini *et al.*, Jul. 1999, J. Bact. 181(13): 4062-4070, incorporated herein by reference). Once the transposon is constructed, it is transposed onto pKOS35-178 to create pKOS35-191. This plasmid is introduced into *Myxococcus xanthus* as described above. This strain contains all the epothilone genes as well as all potential promoters. This strain is fermented and tested for the production of epothilones A and B.

Once the epothilone genes have been established in a strain of *Myxococcus xanthus*, manipulation of any part of the gene cluster, such as changing promoters or swapping modules, can be performed using the kanamycin resistance and galK cassette.

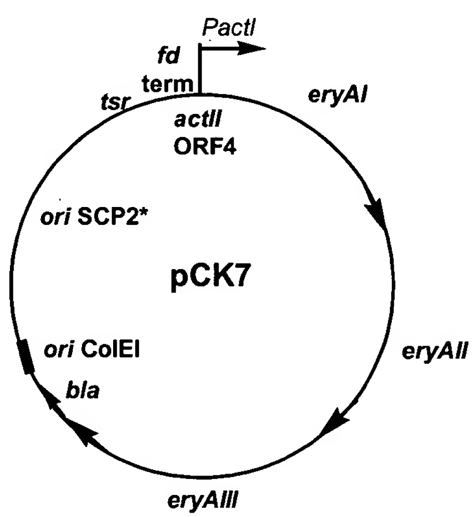
Cultures of *Myxococcus xanthus* containing the epo genes are grown in a number of media and examined for production of epothilones. If the levels of production of epothilones (in particular B or D) are too low to permit large scale fermentation, the

M. xanthus-producing clones are subjected to media development and strain improvement, as described below for enhancing production in *Streptomyces*.

Example 4

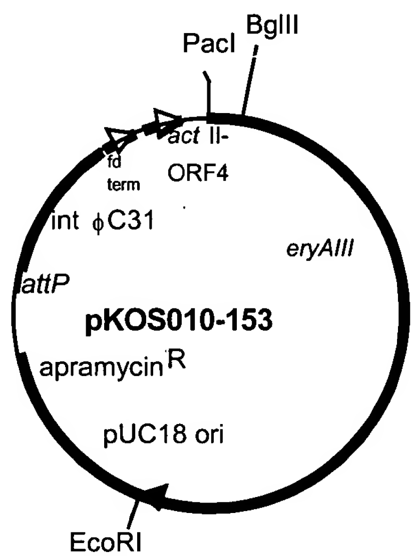
Construction of a *Streptomyces* Expression Vector

The present invention provides recombinant expression vectors for the heterologous expression of modular polyketide synthase genes in *Streptomyces* hosts. These vectors include expression vectors that employ the actI promoter that is regulated by the gene actII ORF4 to allow regulated expression at high levels when growing cells enter stationary phase. Among the vectors available are plasmids pRM1 and pRM5, and derivatives thereof such as pCK7, which are stable, low copy plasmids that carry the marker for thiostrepton resistance in actinomycetes. Such plasmids can accommodate large inserts of cloned DNA and have been used for the expression of the DEBS PKS in *S. coelicolor* and *S. lividans*, the picromycin PKS genes in *S. lividans*, and the oleandomycin PKS genes in *S. lividans*. See U.S. Patent No. 5,712,146. Those of skill in the art recognize that *S. lividans* does not make the tRNA that recognizes the TTA codon for leucine until late-stage growth and that if production of a protein is desired earlier, then appropriate codon modifications can be made.



Plasmid pCK7

Another vector is a derivative of plasmid pSET152 and comprises the actII ORF4-PactI expression system but carries the selectable marker for apramycin resistance. These vectors contain the attP site and integrase gene of the actinophage phiC31 and do not replicate autonomously in *Streptomyces* hosts but integrate by site specific recombination into the chromosome at the attachment site for phiC31 after introduction into the cell. Derivatives of pCK7 and pSET152 have been used together for the heterologous production of a polyketide, with different PKS genes expressed from each plasmid. See U.S. patent application Serial No. 60/129,731, filed 16 Apr. 1999, incorporated herein by reference.



Plasmid pKOS010-153, a pSET152 Derivative

The need to develop expression vectors for the epothilone PKS that function in *Streptomyces* is significant. The epothilone compounds are currently produced in the slow growing, genetically intractable host *Sorangium cellulosum* or are made synthetically. The streptomycetes, bacteria that produce more than 70% of all known antibiotics and important complex polyketides, are excellent hosts for production of epothilones and epothilone derivatives. *S. lividans* and *S. coelicolor* have been developed for the expression of heterologous PKS systems. These organisms can stably maintain cloned heterologous PKS genes, express them at high levels under controlled conditions, and modify the corresponding PKS proteins (e.g. phosphopantetheinylation) so that they

are capable of production of the polyketide they encode. Furthermore, these hosts contain the necessary pathways to produce the substrates required for polyketide synthesis, e.g. malonyl CoA and methylmalonyl CoA. A wide variety of cloning and expression vectors are available for these hosts, as are methods for the introduction and stable maintenance of large segments of foreign DNA. Relative to the slow growing *Sorangium* host, *S. lividans* and *S. coelicolor* grow well on a number of media and have been adapted for high level production of polyketides in fermentors. A number of approaches are available for yield improvements, including rational approaches to increase expression rates, increase precursor supply, etc. Empirical methods to increase the titers of the polyketides, long since proven effective for numerous other polyketides produced in streptomycetes, can also be employed for the epothilone and epothilone derivative producing host cells of the invention.

To produce epothilones by fermentation in a heterologous *Streptomyces* host, the epothilone PKS (including the NRPS module) genes are cloned in two segments in derivatives of pCK7 (loading domain through module 6) and pKOS010-153 (modules 7 through 9). The two plasmids are introduced into *S. lividans* employing selection for thiostrepton and apramycin resistance. In this arrangement, the pCK7 derivative replicates autonomously whereas the pKOS010-153 derivative is integrated in the chromosome. In both vectors, expression of the epothilone genes is from the actI promoter resident within the plasmid.

To facilitate the cloning, the two epothilone PKS encoding segments (one for the loading domain through module six and one for modules seven through nine) were cloned as translational fusions with the N-terminal segment of the KS domain of module 5 of the ery PKS. High level expression has been demonstrated from this promoter employing KS5 as the first translated sequence, see Jacobsen *et al.*, 1998, Biochemistry 37: 4928-4934, incorporated herein by reference. A convenient BsaBI site is contained within the DNA segment encoding the amino acid sequence EPIAV that is highly conserved in many KS domains including the KS-encoding regions of *epoA* and of module 7 in *epoE*.

The expression vector for the loading domain and modules one through six of the epothilone PKS was designated pKOS039-124, and the expression vector for modules seven through nine was designated pKOS039-126. Those of skill in the art will recognize that other vectors and vector components can be used to make equivalent vectors. Because preferred expression vectors of the invention, described below and derived from pKOS039-124 and pKOS039-126, have been deposited under the terms of the Budapest Treaty, only a summary of the construction of plasmids pKOS039-124 and pKOS039-126 is provided below.

The eryKS5 linker coding sequences were cloned as an ~0.4 kb PacI-BglII restriction fragment from plasmid pKOS10-153 into pKOS039-98 to construct plasmid pKOS039-117. The coding sequences for the eryKS5 linker were linked to those for the epothilone loading domain by inserting the ~8.7 kb EcoRI-XbaI restriction fragment from cosmid pKOS35-70.1A2 into EcoRI-XbaI digested plasmid pLItmus28. The ~3.4 kb of BsaBI-NotI and ~3.7 kb NotI-HindIII restriction fragments from the resulting plasmid were inserted into BsaBI-HindIII digested plasmid pKOS039-117 to construct plasmid pKOS039-120. The ~7 kb PacI-XbaI restriction fragment of plasmid pKOS039-120 was inserted into plasmid pKAO18' to construct plasmid pKOS039-123. The final pKOS039-124 expression vector was constructed by ligating the ~34 kb XbaI-AvrII restriction fragment of cosmid pKOS35-70.1A2 with the ~21.1 kb AvrII-XbaI restriction fragment of pKOS039-123..

The plasmid pKOS039-126 expression vector was constructed as follows. First the coding sequences for module 7 were linked from cosmids pKOS35-70.4 and pKOS35-79.85 by cloning the ~6.9 kb BglII-NotI restriction fragment of pKOS35-70.4 and the ~5.9 kb NotI-HindIII restriction fragment of pKOS35-79.85 into BglII-HindIII digested plasmid pLitmus28 to construct plasmid pKOS039-119. The ~12 kb NdeI-NheI restriction fragment of cosmid pKOS35-79.85 was cloned into NdeI-XbaI digested plasmid pKOS039-119 to construct plasmid pKOS039-122.

To fuse the eryKS5 linker coding sequences with the coding sequences for module 7, the ~1 kb BsaBI-BglII restriction fragment derived from cosmid pKOS35-70.4 was cloned into BsaBI-BclI digested plasmid pKOS039-117 to construct plasmid

pKOS039-121. The ~21.5 kb AvrII restriction fragment from plasmid pKOS039-122 was cloned into AvrII-XbaI digested plasmid pKOS039-121 to construct plasmid pKOS039-125. The ~21.8 kb PacI-EcoRI restriction fragment of plasmid pKOS039-125 was ligated with the ~9 kb PacI-EcoRI restriction fragment of plasmid pKOS039-44 to construct pKOS039-126.

Plasmids pKOS039-124 and pKOS126 were introduced into *S. lividans* K4-114 sequentially employing selection for the corresponding drug resistance marker. Because plasmid pKOS039-126 does not replicate autonomously in streptomycetes, the selection is for cells in which the plasmid has integrated in the chromosome by site-specific recombination at the attB site of phiC31. Because the plasmid stably integrates, continued selection for apramycin resistance is not required. Selection can be maintained if desired. The presence of thiostrepton in the medium is maintained to ensure continued selection for plasmid pKOS039-124. Plasmids pKOS039-124 and pKOS039-126 were transformed into *Streptomyces lividans* K4-114, and transformants containing the plasmids were cultured and tested for production of epothilones. Initial tests did not indicate the presence of an epothilone.

sub B9

To improve production of epothilones from these vectors, the eryKS5 linker sequences were replaced by epothilone PKS gene coding sequences, and the vectors were introduced into *Streptomyces coelicolor* CH999. To amplify by PCR coding sequences from the *epoA* gene coding sequence, two oligonucleotides primers were used:
N39-73, 5'-GCTTAATTAAGGAGGACACATATGCCCGTCGTGGCGGATCGTCC-3'; and
N39-74, 5'-GCGGATCCTCGAATCACCGCCAATATC-3'.
The template DNA was derived from cosmid pKOS35-70.8A3. The ~0.8 kb PCR product was digested with restriction enzymes PacI and BamHI and then ligated with the ~2.4 kb BamHI-NotI and the ~6.4 kb PacI-NotI restriction fragments of plasmid pKOS039-120 to construct plasmid pKOS039-136. To make the expression vector for the *epoA*, *epoB*, *epoC*, and *epoD* genes, the ~5 kb PacI-AvrII restriction fragment of plasmid pKOS039-136 was ligated with the ~50 kb PacI-AvrII restriction fragment of plasmid pKOS039-124 to construct the expression plasmid pKOS039-124R. Plasmid pKOS039-124R has

a

been deposited with the ATCC under the terms of the Budapest Treaty and is available under accession ~~number~~ number PTA-926 ______.

sub B10

To amplify by PCR sequences from the *epoE* gene coding sequence, two oligonucleotide primers were used:

`N39-67A, 5'-GCTTAATTAAGGAGGACACATATGACCGACCGAGAAGGCCAGCTC-CTGGA-3', and`
`N39-68, 5'-GGACCTAGGCGGGATGCCGGCGTCT-3'.`

The template DNA was derived from cosmid pKOS35-70.1A2. The ~0.4 kb amplification product was digested with restriction enzymes PacI and AvrII and ligated with either the ~29.5 kb PacI-AvrII restriction fragment of plasmid pKOS039-126 or the ~23.8 kb PacI-AvrII restriction fragment of plasmid pKOS039-125 to construct plasmid pKOS039-126R or plasmid pKOS039-125R, respectively. Plasmid pKOS039-126R was deposited with the ATCC under the terms of the Budapest Treaty and is available under accession ~~number~~ number PTA 927 ______.

a

The plasmid pair pKOS039-124R and pKOS039-126R (as well as the plasmid pair pKOS039-124 and pKOS039-126) contain the full complement of *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, *epoF*, *epoK*, and *epoL* genes. The latter two genes are present on plasmid pKOS039-126R (as well as plasmid pKOS039-126); however, to ensure that these genes were expressed at high levels, another expression vector of the invention, plasmid pKOS039-141 (Figure 8), was constructed in which the *epoK* and *epoL* genes were placed under the control of the ermE* promoter.

sub B11

The *epoK* gene sequences were amplified by PCR using the oligonucleotide primers:

`N39-69, 5'-AGGCATGCATATGACCCAGGAGCAAGCGAATCAGAGTG-3'; and`
`N39-70, 5'-CCAAGCTTTATCCAGCTTTGGAGGGCTTCAAG-3'.`

sub B12

The *epoL* gene sequences were amplified by PCR using the oligonucleotide primers:

`N39-71A, 5'-GTAAGCTTAGGAGGACACATATGATGCAACTCGCGCGCGGGTG-3'; and`
`N39-72, 5'-GCCTGCAGGCTCAGGCTTGCGCAGAGCGT-3'.`

The template DNA for the amplifications was derived from cosmid pKOS35-79.85. The PCR products were subcloned into PCR-script for sequence analysis. Then,

the *epoK* and *epoL* genes were isolated from the clones as NdeI-HindIII and HindIII-EcoRI restriction fragments, respectively, and ligated with the ~6 kb NdeI-EcoRI restriction fragment of plasmid pKOS039-134B, which contains the ermE* promoter, to construct plasmid pKOS039-140. The ~2.4 kb NheI-PstI restriction fragment of plasmid pKOS039-140 was cloned into XbaI-PstI digested plasmid pSAM-Hyg, a plasmid pSAM2 derivative containing a hygromycin resistance conferring gene, to construct plasmid pKOS039-141.

Another variant of plasmid pKOS039-126R was constructed to provide the *epoE* and *epoF* genes on an expression vector without the *epoK* and *epoL* genes. This plasmid, pKOS045-12 (Figure 9), was constructed as follows. Plasmid pXH106 (described in J. Bact., 1991, 173: 5573-5577, incorporated herein by reference) was digested with restriction enzymes StuI and BamHI, and the ~2.8 kb restriction fragment containing the xylE and hygromycin resistance conferring genes was isolated and cloned into EcoRV-BglII digested plasmid pLitmus28. The ~2.8 kb NcoI-AvrII restriction fragment of the resulting plasmid was ligated to the ~18 kb PacI-BspHI restriction fragment of plasmid pKOS039-125R and the ~9 kb SpeI-PacI restriction fragment of plasmid pKOS039-42 to construct plasmid pKOS045-12.

To construct an expression vector that comprised only the *epoL* gene, plasmid pKOS039-141 was partially digested with restriction enzyme NdeI, the ~9 kb NdeI restriction fragment was isolated, and the fragment then circularized by ligation to yield plasmid pKOS039-150.

The various expression vectors described above were then transformed into *Streptomyces coelicolor* CH999 and *S. lividans* K4-114 in a variety of combinations, the transformed host cells fermented on plates and in liquid culture (R5 medium, which is identical to R2YE medium without agar). Typical fermentation conditions follow. First, a seed culture of about 5 mL containing 50 μg/L thiostrepton was inoculated and grown at 30°C for two days. Then, about 1 to 2 mL of the seed culture was used to inoculate a production culture of about 50 mL containing 50 μg/L thiostrepton and 1 mM cysteine, and the production culture was grown at 30°C for 5 days. Also, the seed culture was used

to prepare plates of cells (the plates contained the same media as the production culture with 10 mM propionate), which were grown at 30°C for nine days.

Certain of the *Streptomyces coelicolor* cultures and culture broths were analyzed for production of epothilones. The liquid cultures were extracted with three times with equal volumes of ethyl acetate, the organic extracts combined and evaporated, and the residue dissolved in acetonitrile for LC/MS analysis. The agar plate media was chopped and extracted twice with equal volumes of acetone, and the acetone extracts were combined and evaporated to an aqueous slurry, which was extracted three times with equal volumes of ethyl acetate. The organic extracts were combined and evaporated, and the residue dissolved in acetonitrile for LC/MS analysis.

Production of epothilones was assessed using LC-mass spectrometry. The output flow from the UV detector of an analytical HPLC was split equally between a Perkin-Elmer/Sciex API100LC mass spectrometer and an Alltech 500 evaporative light scattering detector. Samples were injected onto a 4.6 x 150 mm reversed phase HPLC column (MetaChem 5 m ODS-3 Inertsil) equilibrated in water with a flow rate of 1.0 mL/min. UV detection was set at 250 nm. Sample components were separated using H2O for 1 minute, then a linear gradient from 0 to 100% acetonitrile over 10 minutes. Under these conditions, epothilone A elutes at 10.2 minutes and epothilone B elutes at 10.5 minutes. The identity of these compounds was confirmed by the mass spectra obtained using an atmospheric chemical ionization source with orifice and ring voltages set at 75 V and 300 V, respectively, and a mass resolution of 0.1 amu. Under these conditions, epothilone A shows [M+H] at 494.4 amu, with observed fragments at 476.4, 318.3, and 306.4 amu. Epothilone B shows [M+H] at 508.4 amu, with observed fragments at 490.4, 320.3, and 302.4 amu.

Transformants containing the vector pairs pKOS039-124R and pKOS039-126R or pKOS039-124 and pKOS039-126R produced detectable amounts of epothilones A and B. Transformants containing these plasmid pairs and the additional plasmid pKOS039-141 produced similar amounts of epothilones A and B, indicating that the additional copies of the *epoK* and *epoL* genes were not required for production under the test conditions employed. Thus, these transformants produced epothilones A and B when recombinant

epoA, *epoB*, *epoC*, *epoD*, *epoE*, *epoF*, *epoK*, and *epoL* genes were present. In some cultures, it was observed that the absence of propionate increased the proportion of epothilone B to epothilone A.

Transformants containing the plasmid pair pKOS039-124R and pKOS045-12 produced epothilones C and D, as did transformants containing this plasmid pair and the additional plasmid pKOS039-150. These results showed that the *epoL* gene was not required under the test conditions employed to form the C-12-C-13 double bond. These results indicate that either the epothilone PKS gene alone is able to form the double bond or that *Streptomyces coelicolor* expresses a gene product able to convert epothilones G and H to epothilones C and D. Thus, these transformants produced epothilones C and D when recombinant *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* genes were present.

The heterologous expression of the epothilone PKS described herein is believed to represent the recombinant expression of the largest proteins and active enzyme complex that have ever been expressed in a recombinant host cell. The epothilone producing *Streptomyces coelicolor* transformants exhibited growth characteristics indicating that either the epothilone PKS genes, or their products, or the epothilones inhibited cell growth or were somewhat toxic to the cells. Any such inhibition or toxicity could be due to accumulation of the epothilones in the cell, and it is believed that the native *Sorangium* producer cells may contain transporter proteins that in effect pump epothilones out of the cell. Such transporter genes are believed to be included among the ORFs located downstream of the *epoK* gene and described above. Thus, the present invention provides *Streptomyces* and other host cells that include recombinant genes that encode the products of one or more, including all, of the ORFs in this region.

For example, each ORF can be cloned behind the ermE* promoter, see Stassi *et al.*, 1998, Appl. Microbiol. Biotechnol. 49: 725-731, incorporated herein by reference, in a pSAM2-based plasmid that can integrate into the chromosome of *Streptomyces coelicolor* and *S. lividans* at a site distinct from attB of phage phiC31, see Smokvina *et al.*, 1990, Gene 94: 53-59, incorporated herein by reference. A pSAM2-based vector carrying the gene for hygromycin resistance is modified to carry the ermE* promoter

along with additional cloning sites. Each ORF downstream is PCR cloned into the vector which is then introduced into the host cell (also containing pKOS039-124R and pKOS039-126R or other expression vectors of the invention) employing hygromycin selection. Clones carrying each individual gene downstream from *epoK* are analyzed for increased production of epothilones.

Additional fermentation and strain improvement efforts can be conducted as illustrated by the following. The levels of expression of the PKS genes in the various constructs can be measured by assaying the levels of the corresponding mRNAs (by quantitative RT PCR) relative to the levels of another heterologous PKS mRNA (e.g. picromycin) produced from genes cloned in similar expression vectors in the same host. If one of the epothilone transcripts is underproduced, experiments to enhance its production by cloning the corresponding DNA segment in a different expression vector are conducted. for example, multiple copies of any one or more of the epothilone PKS genes can be introduced into a cell if one or more gene products are rate limiting for biosynthesis. If the basis for low level production is not related to low level PKS gene expression (at the RNA level), an empirical mutagenesis and screening approach that is the backbone of yield improvement of every commercially important fermentation product is undertaken. Spores are subjected to UV, X-ray or chemical mutagens, and individual survivors are plated and picked and tested for the level of compound produced in small scale fermentations. Although this process can be automated, one can examine several thousand isolates for quantifiable epothilone production using the susceptible fungus *Mucor hiemalis* as a test organism.

Sub B13

Another method to increase the yield of epothilones produced is to change the KS^Y domain of the loading domain of the epothilone PKS to a KS^Q domain. Such altered loading domains can be constructed in any of a variety of ways, but one illustrative method follows. Plasmid pKOS39-124R of the invention can be conveniently used as a starting material. To amplify DNA fragments useful in the construction, four oligonucleotide primers are employed:

```
N39-83: 5'-CCGGTATCCACCGCGACACACGGC-3',
N39-84: 5'-GCCAGTCGTCCTCGCTCGTGGCCGTTC-3',
```

and N39-73 and N39-74, which have been described above. The PCR fragment generated with N37-73 and N39-83 and the PCR fragment generated with N39-74 and N39-84 are treated with restriction enzymes PacI and BamHI, respectively, and ligated with the ~3.1 kb PacI-BamHI fragment of plasmid pKOS39-120 to construct plasmid pKOS039-148. The ~0.8 kb PacI-BamHI restriction fragment of plasmid pKOS039-148 (comprising the two PCR amplification products) is ligated with the ~2.4 kb BamHI-NotI restriction fragment and the ~6.4 kb PacI-NotI restriction fragment of plasmid pKOS39-120 to construct pKOS39-136Q. The ~5 kb PacI-AvrII restriction fragment of plasmid pKOS039-136Q is ligated to the ~50 kb PacI-AvrII restriction fragment of plasmid pKOS039-124 to construct plasmid pKOS39-124Q. Plasmids pKOS039-124Q and pKOS039-126R are then transformed into *Streptomyces coelicolor* CH999 for epothilone production.

The *epoA* through *epoF*, optionally with *epoK* or with *epoK* plus *epoL*, genes cloned and expressed are sufficient for the synthesis of epothilone compounds, and the distribution of the C-12 H to C-12 methyl congeners appears to be similar to that seen in the natural host (A:B::2:1). This ratio reflects that the AT domain of module 4 more closely resembles that of the malonyl rather than methylmalonyl specifying AT consensus domains. Thus, epothilones D and B are produced at lower quantities than their C-12 unmethylated counterparts C and A. The invention provides PKS genes that produce epothilone D and/or B exclusively. Specifically, methylmalonyl CoA specifying AT domains from a number of sources (e.g. the narbonolide PKS, the rapamycin PKS, and others listed above) can be used to replace the naturally occurring at domain in module 4. The exchange is performed by direct cloning of the incoming DNA into the appropriate site in the epothilone PKS encoding DNA segment or by gene replacement through homologous recombination.

For gene replacement through homologous recombination, the donor sequence to be exchanged is placed in a delivery vector between segments of at least 1 kb in length that flank the AT domain of epo module 4 encoding DNA. Crossovers in the homologous regions result in the exchange of the epo AT4 domain with that on the delivery vector. Because pKOS039-124 and pKOS039-124R contain AT4 coding sequences, they can be

used as the host DNA for replacement. The adjacent DNA segments are cloned in one of a number of *E. coli* plasmids that are temperature sensitive for replication. The heterologous AT domains can be cloned in these plasmids in the correct orientation between the homologous regions as cassettes enabling the ability to perform several AT exchanges simultaneously. The reconstructed plasmid (pKOS039-124* or pKOS039-124R*) is tested for ability to direct the synthesis of epothilone B and/or by introducing it along with pKOS039-126 or pKOS039-126R in *Streptomyces coelicolor* and/or *S. lividans*.

Because the titers of the polyketide can vary from strain to strain carrying the different gene replacements, the invention provides a number of heterologous methylmalonyl CoA specifying AT domains to ensure that production of epothilone D at titers equivalent to that of the C and D mixture produced in the *Streptomyces coelicolor* host described above. In addition, larger segments of the donor genes can be used for the replacements, including, in addition to the AT domain, adjacent upstream and downstream sequences that correspond to an entire module. If an entire module is used for the replacement, the KS, methylmalonyl AT, DH, KR, ACP – encoding DNA segment can be obtained from for example and without limitation the DNA encoding the tenth module of the rapamycin PKS, or the first or fifth modules of the FK-520 PKS.

Example 5

Heterologous Expression of EpoK and Conversion of Epothilone D to Epothilone B

This Example describes the construction of *E. coli* expression vectors for *epoK*. The *epoK* gene product was expressed in *E. coli* as a fusion protein with a polyhistidine tag (his tag). The fusion protein was purified and used to convert epothilone D to epothilone B.

Plasmids were constructed to encode fusion proteins composed of six histidine residues fused to either the amino or carboxy terminus of EpoK. The following oligos were used to construct the plasmids:

Sub B14

55-101.a-1:
5'-AAAAACATATGCACCACCACCACCACCACATGACACAGGAGCAAGCGAAT-CAGAGTGAG-3',

55-101.b:
5'-AAAAAGGATCCTTAATCCAGCTTTGGAGGGCTT-3',
55-101.c:
5'-AAAAACATATGACACAGGAGCAAGCGAAT-3', and
55-101.d:
5'-AAAAAGGATCCTTAGTGGTGGTGGTGGTGGTGTCCAGCTTTGGAGGGCTTC-AAGATGAC-3'.

The plasmid encoding the amino terminal his tag fusion protein, pKOS55-121, was constructed using primers 55-101.a-1and 55-101.b, and the one encoding the carboxy terminal his tag, pKOS55-129, was constructed using primers 55-101.c and 55-101.d in PCR reactions containing pKOS35-83.5 as the template DNA. Plasmid pKOS35-83.5 contains the ~5 kb NotI fragment comprising the *epoK* gene ligated into pBluescriptSKII+ (Stratagene). The PCR products were cleaved with restriction enzymes BamHI and NdeI and ligated into the BamHI and NdeI sites of pET22b (Invitrogen). Both plasmids were sequenced to verify that no mutations were introduced during the PCR amplification. Protein gels were run as known in the art.

Purification of EpoK was performed as follows. Plasmids pKOS55-121 and pKOS55-129 were transformed into BL21(DE3) containing the groELS expressing plasmid pREP4-groELS (Caspers *et al.*, 1994, Cellular and Molecular Biology 40(5): 635-644). The strains were inoculated into 250 mL of M9 medium supplemented with 2 mM MgSO4, 1% glucose, 20 mg thiamin, 5 mg $FeCl_2$, 4 mg $CaCl_2$ and 50 mg levulinic acid. The cultures were grown to an OD_{600} between 0.4 and 0.6, at which point IPTG was added to 1 mM, and the cultures were allowed to grow for an additional two hours. The cells were harvested and frozen at -80°C. The frozen cells were resuspended in 10 ml of buffer 1 (5 mM imidazole, 500 mM NaCl, and 45 mM Tris pH 7.6) and were lysed by sonicating three times for 15 seconds each on setting 8. The cellular debris was pelleted by spinning in an SS-34 rotor at 16,000 rpm for 30 minutes. The supernatant was removed and spun again at 16,000 rpm for 30 minutes. The supernatant was loaded onto a 5 mL nickel column (Novagen), after which the column was washed with 50 mL of buffer 1 (Novagen). EpoK was eluted with a gradient from 5 mM to 1M imidazole. Fractions containing EpoK were pooled and dialyzed twice against 1 L of dialysis buffer (45 mM Tris pH7.6, 0.2 mM DTT, 0.1 mM EDTA, and 20% glycerol). Aliquots were

frozen in liquid nitrogen and stored at -80°C. The protein preparations were greater than 90% pure.

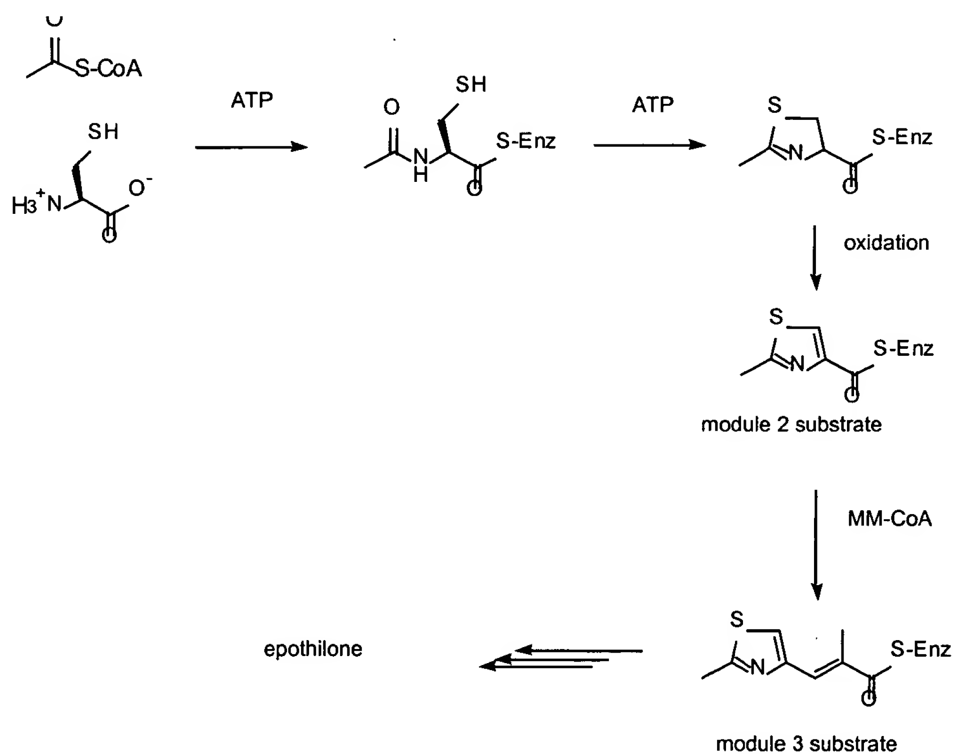
The EpoK assay was performed as follows (See Betlach *et al.*, *Biochem* (1998) 37:14937, incorporated herein by reference). Briefly, reactions consisted of 50 mM Tris (pH7.5), 21 μM spinach ferredoxin, 0.132 units of spinach ferredoxin: $NADP^+$ oxidoreductase, 0.8 units of glucose-6-phosphate dehydrogenase, 1.4 mM NADP, and 7.1 mM glucose-6-phosphate, 100 μM or 200 μM epothilone D (a generous gift of S. Danishefsky), and 1.7 μM amino terminal his tagged EpoK or 1.6 μM carboxy terminal his tagged EpoK in a 100 μL volume. The reactions were incubated at 30°C for 67 minutes and stopped by heating at 90°C for 2 minutes. The insoluble material was removed by centrifugation, and 50 μL of the supernatant were analyzed by LC/MS. HPLC conditions: Metachem 5 *μ* ODS-3 Inertsil (4.6 X 150 mm); 80% H_2O for 1 min, then to 100% MeCN over 10 min at 1 mL/min, with UV (λ_{max}=250 nm), ELSD, and MS detection. Under these conditions, epothilone D eluted at 11.6 min and epothilone B at 9.3 min. the LC/MS spectra were obtained using an atmosphere pressure chemical ionization source with orifice and ring voltages set at 20 V and 250 V, respectively, at a mass resolution of 1 amu. Under these conditions, epothilone E shows an [M+H] at *m/z* 493, with observed fragments at 405 and 304. Epothilone B shows an [M+H] at *m/z* 509, with observed fragments at 491 and 320.

The reactions containing EpoK and epothilone D contained a compound absent in the control that displayed the same retention time, molecular weight, and mass fragmentation pattern as pure epothilone B. With an epothilone D concentration of 100 μM, the amino and the carboxy terminal his tagged EpoK was able to convert 82% and 58% to epothilone B, respectively. In the presence of 200 μM, conversion was 44% and 21%, respectively. These results demonstrate that EpoK can convert epothilone D to epothilone B.

Example 6

Modified Epothilones from Chemobiosynthesis

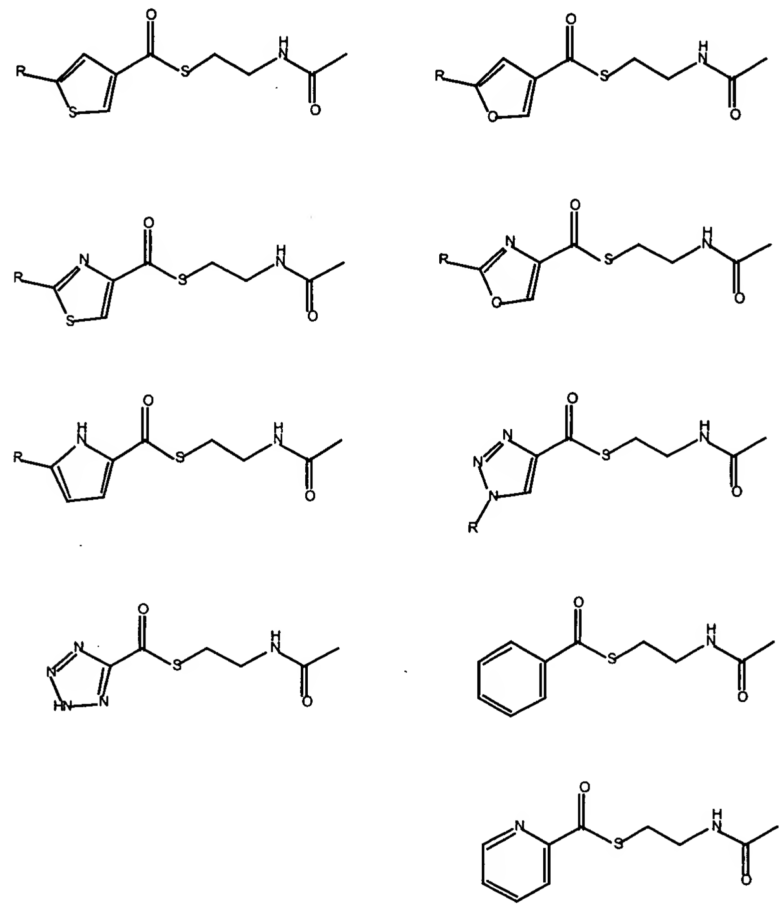
This Example describes a series of thioesters provided by the invention for production of epothilone derivatives via chemobiosynthesis. The DNA sequence of the biosynthetic gene cluster for epothilone from *Sorangium cellulosum* indicates that priming of the PKS involves a mixture of polyketide and amino acid components. Priming involves loading of the PKS-like portion of the loading domain with malonyl CoA followed by decarboxylation and loading of the module one NRPS with cysteine, then condensation to form enzyme-bound N-acetylcysteine. Cyclization to form a thiazoline is followed by oxidation to form enzyme bound 2-methylthiazole-4-carboxylate, the product of the loading domain and NRPS. Subsequent condensation with methylmalonyl CoA by the ketosynthase of module 2 provides the substrate for module, as shown in the following diagram.



The present invention provides methods and reagents for chemobiosynthesis to produce epothilone derivatives in a manner similar to that described to make 6-dEB and

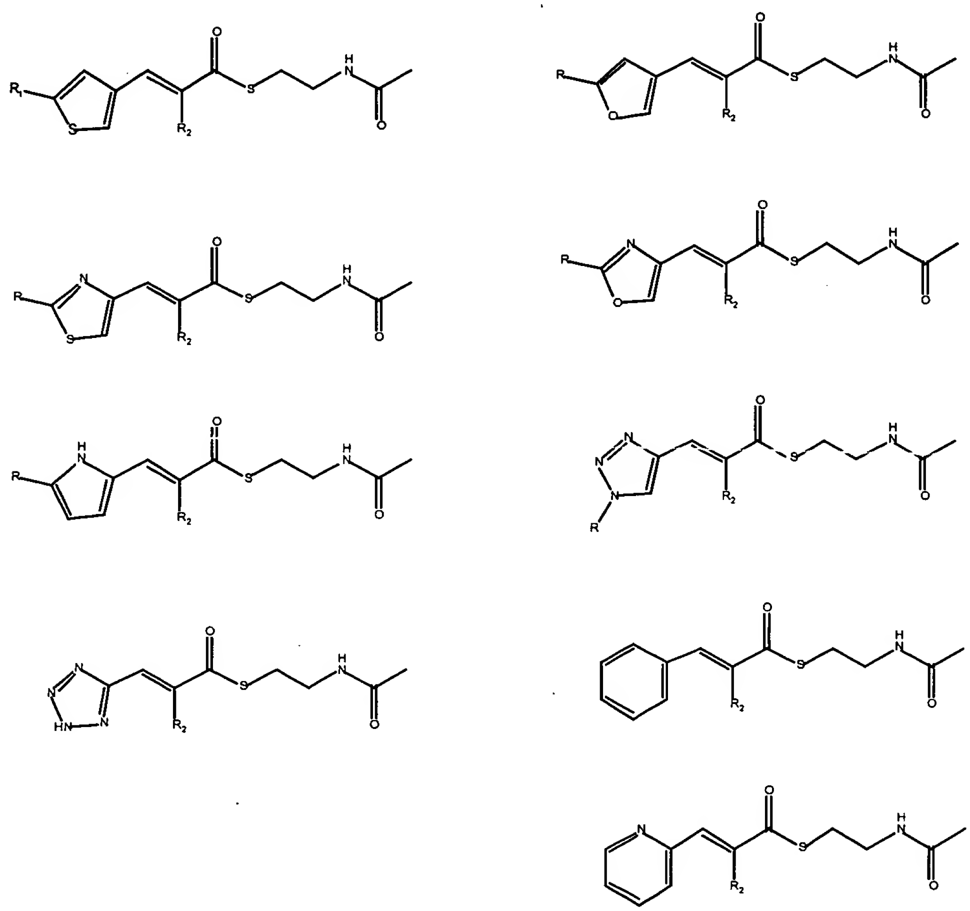
erythromycin analogs in PCT Pat. Pub. Nos. 99/03986 and 97/02358. Two types of feeding substrates are provided: analogs of the NRPS product, and analogs of the module 3 substrate. The module 2 substrates are used with PKS enzymes with a mutated NRPS-like domain, and the module 3 substrates are used with PKS enzymes with a mutated KS domain in module 2.

The following illustrate module 2 substrates (as N-acetyl cysteamine thioesters) for use as substrates for epothilone PKS with modified inactivated NRPS:



The module 2 substrates are prepared by activation of the corresponding carboxylic acid and treatment with N-acetylcysteamine. Activation methods include formation of the acid chloride, formation of a mixed anhydride, or reaction with a condensing reagent such as a carbodiimide.

Exemplary module 3 substrates, also as NAc thioesters for use as substrates for epothilone PKS with KS2 knockout are:



These compounds are prepared in a three-step process. First, the appropriate aldehyde is treated with a Wittig reagent or equivalent to form the substituted acrylic ester. The ester is saponified to the acid, which is then activated and treated with N-acetylcysteamine.

Illustrative reaction schemes for making module 2 and module 3 substrates follow. Additional compounds suitable for making starting materials for polyketide synthesis by the epothilone PKS are shown in Figure 2 as carboxylic acids (or aldehydes that can be converted to carboxylic acids) that are converted to the N-acylcysteamides for supplying to the host cells of the invention.

A. Thiophene-3-carboxylate N-acetylcysteamine thioester

A solution of thiophene-3-carboxylic acid (128 mg) in 2 mL of dry tetrahydrofuran under inert atmosphere was treated with triethylamine (0.25 mL) and diphenylphosphoryl azide (0.50 mL). After 1 hour, N-acetylcysteamine (0.25 mL) was added, and the reaction was allowed to proceed for 12 hours. The mixture was poured into water and extracted three times with equal volumes of ethyl acetate. The organic extracts were combined, washed sequentially with water, 1 N HCl, sat. $CuSO_4$, and brine, then dried over $MgSO_4$, filtered, and concentrated under vacuum. Chromatography on SiO_2 using ether followed by ethyl acetate provided pure product, which crystallized upon standing.

B. Furan-3-carboxylate N-acetylcysteamine thioester

A solution of furan-3-carboxylic acid (112 mg) in 2 mL of dry tetrahydrofuran under inert atmosphere was treated with triethylamine (0.25 mL) and diphenylphosphoryl azide (0.50 mL). After 1 hour, N-acetylcysteamine (0.25 mL) was added and the reaction was allowed to proceed for 12 hours. The mixture was poured into water and extracted three times with equal volumes of ethyl acetate. The organic extracts were combined, washed sequentially with water, 1 N HCl, sat. $CuSO_4$, and brine, then dried over $MgSO_4$, filtered, and concentrated under vacuum. Chromatography on SiO_2 using ether followed by ethyl acetate provided pure product, which crystallized upon standing.

C. Pyrrole-2-carboxylate N-acetylcysteamine thioester

A solution of pyrrole-2-carboxylic acid (112 mg) in 2 mL of dry tetrahydrofuran under inert atmosphere was treated with triethylamine (0.25 mL) and diphenylphosphoryl azide (0.50 mL). After 1 hour, N-acetylcysteamine (0.25 mL) was added and the reaction was allowed to proceed for 12 hours. The mixture was poured into water and extracted three times with equal volumes of ethyl acetate. The organic extracts were combined, washed sequentially with water, 1 N HCl, sat. $CuSO_4$, and brine, then dried over $MgSO_4$,filtered, and concentrated under vacuum. Chromatography on SiO_2 using ether followed by ethyl acetate provided pure product, which crystallized upon standing.

D. 2-Methyl-3-(3-thienyl)acrylate N-acetylcysteamine thioester

(1) Ethyl 2-methyl-3-(3-thienyl)acrylate: A mixture of thiophene-3-carboxaldehyde (1.12 g) and (carbethoxyethylidene)triphenylphosphorane (4.3 g) in dry tetrahydrofuran (20 mL) was heated at reflux for 16 hours. The mixture was cooled to ambient temperature and concentrated to dryness under vacuum. The solid residue was suspended in 1:1 ether/hexane and filtered to remove triphenylphosphine oxide. The filtrate was filtered through a pad of SiO_2 using 1:1 ether/hexane to provide the product (1.78 g, 91%) as a pale yellow oil.

(2) 2-Methyl-3-(3-thienyl)acrylic acid: The ester from (1) was dissolved in a mixture of methanol (5 mL) and 8 N KOH (5 mL) and heated at reflux for 30 minutes. The mixture was cooled to ambient temperature, diluted with water, and washed twice with ether. The aqueous phase was acidified using 1N HCl then extracted 3 times with equal volumes of ether. The organic extracts were combined, dried with $MgSO_4$, filtered, and concentrated to dryness under vacuum. Crystallization from 2:1 hexane/ether provided the product as colorless needles.

(3) 2-Methyl-3-(3-thienyl)acrylate N-acetylcysteamine thioester: A solution of 2-Methyl-3-(3-thienyl)acrylic acid (168 mg) in 2 mL of dry tetrahydrofuran under inert atmosphere was treated with triethylamine (0.56 mL) and diphenylphosphoryl azide (0.45 mL). After 15 minutes, N-acetylcysteamine (0.15 mL) is added and the reaction is allowed to proceed for 4 hours. The mixture is poured into water and extracted three times with equal volumes of ethyl acetate. The organic extracts are combined, washed sequentially with water, 1 N HCl, sat. $CuSO_4$, and brine, then dried over $MgSO_4$,filtered, and concentrated under vacuum. Chromatography on SiO_2 using ethyl acetate provided pure product, which crystallized upon standing.

The above compounds are supplied to cultures of host cells containing a recombinant epothilone PKS of the invention in which either the NRPS or the KS domain of module 2 as appropriate has been inactivated by mutation to prepare the corresponding epothilone derivative of the invention.

Example 7

Producing Epothilones and Epothilone Derivatives in *Sorangium cellulosum* SMP44

The present invention provides a variety of recombinant *Sorangium cellulosum* host cells that produce less complex mixtures of epothilones than the naturally occurring epothilone producers as well as host cells that produce epothilone derivatives. This Example illustrates the construction of such strains by describing how to make a strain that produce only epothilones C and D without epothilones A and B. To construct this strain, an inactivating mutation is made in *epoK*. Using plasmid pKOS35-83.5, which contains a NotI fragment harboring the *epoK* gene, the kanamycin and bleomycin resistance markers from Tn5 are ligated into the ScaI site of the *epoK* gene to construct pKOS90-55. The orientation of the resistance markers is such that transcription initiated at the kanamycin promoter drives expression of genes immediately downstream of *epoK*. In other words, the mutation should be nonpolar. Next, the origin of conjugative transfer, oriT, from RP4 is ligated into pKOS90-55 to create pKOS90-63. This plasmid can be introduced into S17-1 and conjugated into SMP44. The transconjugants are selected on phleomycin plates as previously described. Alternatively, electroporation of the plasmid can be achieved using conditions described above for *Myxococcus xanthus*.

Because there are three generalized transducing phages for *Myxococcus xanthus*, one can transfer DNA from *M. xanthus* to SMP44. First, the *epoK* mutation is constructed in *M. xanthus* by linearizing plasmid pKOS90-55 and electroporating into *M. xanthus*. Kanamycin resistant colonies are selected and have a gene replacement of *epoK*. This strain is infected with Mx9, Mx8, Mx4 ts18 hft hrm phages to make phage lysates. These lysates are then individually infected into SMP44 and phleomycin resistant colonies are selected. Once the strain is constructed, standard fermentation procedures, as described below, are employed to produce epothilones C and D.

Prepare a fresh plate of *Sorangium* host cells (dispersed) on S42 medium. S42 medium contains tryptone, 0.5 g/L; $MgSO_4$, 1.5 g/L; HEPES, 12 g/L; agar, 12 g/L, with deionized water. The pH of S42 medium is set to 7.4 with KOH. To prepare S42 medium, after autoclaving at 121°C for at least 30 minutes, add the following ingredients (per liter): $CaCl_2$, 1 g; K_2HPO_4, 0.06 g; Fe Citrate, 0.008 g; Glucose, 3.5 g; Ammonium

sulfate, 0.5 g; Spent liquid medium, 35 mL; and 200 micrograms/mL of kanamycin is added to prevent contamination. Incubate the culture at 32°C for 4-7 days, or until orange sorangia appear on the surface.

To prepare a seed culture for inoculating agar plates/bioreactor, the following protocol is followed. Scrape off a patch of orange *Sorangium* cells from the agar (about 5 mm^2) and transfer to a 250 ml baffle flask with 38 mm silicone foam closures containing 50 ml of Soymeal Medium containing potato starch, 8 g; defatted soybean meal, 2 g; yeast extract, 2 g; Iron (III) sodium salt EDTA, 0.008 g; $MgSO_4.7H_2O$, 1 g; $CaCl_2.2H_2O$, 1 g; glucose, 2 g; HEPES buffer, 11.5 g. Use deionized water, and adjust pH to 7.4 with 10% KOH. Add 2-3 drops of antifoam B to prevent foaming. Incubate in a coffin shaker for 4-5 days at 30°C and 250 RPM. The culture should appear an orange color. This seed culture can be subcultured repeatedly for scale-up to inoculate in the desired volume of production medium.

The same preparation can be used with Medium 1 containing (per liter) $CaCl_2.2H_2O$, 1 g; yeast extract, 2 g; Soytone, 2 g; FeEDTA, 0.008 g; Mg $SO_4.7H_2O$, 1 g; HEPES, 11.5 g. Adjust pH to 7.4 with 10% KOH, and autoclave at 121°C for 30 minutes. Add 8 ml of 40% glucose after sterilization. Instead of a baffle flask, use a 250 ml coiled spring flask with a foil cover. Include 2-3 drops of antifoam B, and incubate in a coffin shaker for 7 days at 37°C and 250 RPM. Subculture the entire 50 mL into 500 mL of fresh medium in a baffled narrow necked Fernbach flask with a 38 mm silicone foam closure. Include 0.5 ml of antifoam to the culture. Incubate under the same conditions for 2-3 days. Use at least a 10% inoculum for a bioreactor fermentation.

To culture on solid media, the following protocol is used. Prepare agar plates containing (per liter of CNS medium) KNO_3, 0.5 g; Na_2HPO_4, 0.25 g; $MgSO_4.7H_2O$, 1 g; $FeCl_2$, 0.01 g; HEPES, 2.4 g; Agar, 15 g; and sterile Whatman filter paper. While the agar is not completely solidified, place a sterile disk of filter paper on the surface. When the plate is dry, add just enough of the seed culture to coat the surface evenly (about 1 mL). Spread evenly with a sterile loop or an applicator, and place in a 32°C incubator for 7 days. Harvest plates.

For production in a 5 L bioreactor, the following protocol is used. The fermentation can be conducted in a B. Braun Biostat MD-1 5L bioreactor. Prepare 4 L of production medium (same as the soymeal medium for the seed culture without HEPES buffer). Add 2% (volume to volume) XAD-16 absorption resin, unwashed and untreated, e.g. add 1 mL of XAD per 50 mL of production medium. Use 2.5 N H_2SO_4 for the acid bottle, 10% KOH for the base bottle, and 50% antifoam B for the antifoam bottle. For the sample port, be sure that the tubing that will come into contact with the culture broth has a small opening to allow the XAD to pass through into the vial for collecting daily samples. Stir the mixture completely before autoclaving to evenly distribute the components. Calibrate the pH probe and test dissolved oxygen probe to ensure proper functioning. Use a small antifoam probe, ~3 inches in length. For the bottles, use tubing that can be sterile welded, but use silicone tubing for the sample port. Make sure all fittings are secure and the tubings are clamped off, not too tightly, with C-clamps. Do not clamp the tubing to the exhaust condenser. Attach 0.2 μm filter disks to any open tubing that is in contact with the air. Use larger ACRO 50 filter disks for larger tubing, such as the exhaust condenser and the air inlet tubing. Prepare a sterile empty bottle for the inoculum. Autoclave at 121°C with a sterilization time of 90 minutes. Once the reactor has been taken out of the autoclave, connect the tubing to the acid, base, and antifoam bottles through their respective pump heads. Release the clamps to these bottles, making sure the tubing has not been welded shut. Attach the temperature probe to the control unit. Allow the reactor to cool, while sparging with air through the air inlet at a low air flow rate.

After ensuring the pumps are working and there is no problem with flow rate or clogging, connect the hoses from the water bath to the water jacket and to the exhaust condenser. Make sure the water jacket is nearly full. Set the temperature to 32°C. Connect pH, D.O., and antifoam probes to the main control unit. Test the antifoam probe for proper functioning. Adjust the set point of the culture to 7.4. Set the agitation to 400 RPM. Calibrate the D.O. probe using air and nitrogen gas. Adjust the airflow using the rate at which the fermentation will operate, e.g. 1 LPM (liter per minute). To control the dissolved oxygen level, adjust the parameters under the cascade setting so that agitation

will compensate for lower levels of air to maintain a D.O. value of 50%. Set the minimum and maximum agitation to 400 and 1000 RPM respectively, based on the settings of the control unit. Adjust the settings, if necessary.

Check the seed culture for any contamination before inoculating the fermenter. The *Sorangium cellulosum* cells are rod shaped like a pill, with 2 large distinct circular vacuoles at opposite ends of the cell. Length is approximately 5 times that of the width of the cell. Use a 10% inoculum (minimum) volume, e.g. 400 mL into 4 L of production medium. Take an initial sample from the vessel and check against the bench pH. If the difference between the fermenter pH and the bench pH is off by ≥ 0.1 units, do a 1 point recalibration. Adjust the deadband to 0.1. Take daily 25 mL samples noting fermenter pH, bench pH, temperature, D.O., airflow, agitation, acid, base, and antifoam levels. Adjust pH if necessary. Allow the fermenter to run for seven days before harvesting.

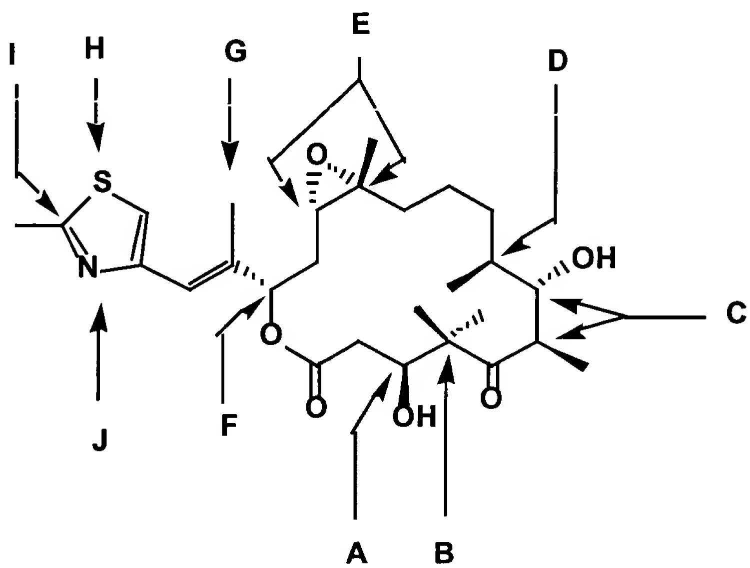
Extraction and analysis of compounds is performed substantially as described above in Example 4. In brief, fermentation culture is extracted twice with ethyl acetate, and the ethyl acetate extract is concentrated to dryness and dissolved/suspended in ~500 μL of MeCN-H_2O (1:1). The sample is loaded onto a 0.5 mL Bakerbond ODS SPE cartridge pre-equilibrated with MeCN-H_2O (1:1). The cartridge is washed with 1 mL of the same solvent, followed by 2 mL of MeCN. The MeCN eluent is concentrated to dryness, and the residue is dissolved in 200 μL of MeCN. Samples (50 μL) are analyzed by HPLC/MS on a system comprised of a Beckman System Gold HPLC and PE Sciex API100LC single quadrapole MS-based detector equipped with an atmospheric pressure chemical ionization source. Ring and orifice voltages are set to 75V and 300V, respectively, and a dual range mass scan from *m/z* 290-330 and 450-550 is used. HPLC conditions: Metachem 5μ ODS-3 Inertsil (4.6 X 150 mm); 100% H_2O for 1 min, then to 100% MeCN over 10 min a 1 mL/min. Epothilone A elutes at 0.2 min under these conditions and gives characteristic ions at *m/z* 494 (M+H), 476 (M+H-H_2O), 318, and 306.

Example 8

Epothilone Derivatives as Anti-Cancer Agents

The novel epothilone derivatives shown below by Formula (1) set forth above are potent anti-cancer agents and can be used for the treatment of patients with various forms of cancer, including but not limited to breast, ovarian, and lung cancers.

The epothilone structure-activity relationships based on tubulin binding assay are (see Nicolaou *et al.*, 1997, Angew. Chem. Int. Ed. Engl. 36: 2097-2103, incorporated herein by reference) are illustrated by the diagram below.



A) (3S) configuration important; B) 4,4-ethano group not tolerated; C) (6R, 7S) configuration crucial; D) (8S) configuration important, 8,8-dimethyl group not tolerated; E) epoxide not essential for tubulin polymerization activity, but may be important for cytotoxicity; epoxide configuration may be important; R group important; both olefin geometries tolerated; F) (15S) configuration important; G) bulkier group reduces activity; H) oxygen substitution tolerated; I) substitution important; J) heterocycle important.

Thus, this SAR indicates that modification of the C1-C8 segment of the molecule can have strong effects on activity, whereas the remainder of the molecule is relatively tolerant to change. Variation of substituent stereochemistry with the C1-C8 segment, or removal of the functionality, can lead to significant loss of activity. Epothilone derivative compounds A-H differ from epothilone by modifications in the less sensitive portion of

the molecule and so possess good biological activity and offer better pharmacokinetic characteristics, having improved lipophilic and steric profiles.

These novel derivatives can be prepared by altering the genes involved in the biosynthesis of epothilone optionally followed by chemical modification. The 9-hydroxy-epothilone derivatives prepared by genetic engineering can be used to generate the carbonate derivatives (compound D) by treatment with triphosgene or 1,1' carbonyldiimidazole in the presence of a base. In a similar manner, the 9,11-dihydroxy-epothilone derivative, upon proper protection of the C-7 hydroxyl group if it is present, yields the carbonate derivatives (compound F). Selective oximation of the 9 oxo-epothilone derivatives with hydroxylamine followed by reduction (Raney nickel in the presence of hydrogen or sodium cyanoborohydride) yield the 9-amino analogs. Reacting these 9-amino derivatives with p-nitrophenyl chloroformate in the presence of base and subsequently reacting with sodium hydride will produce the carbamate derivatives (compound E). Similarly, the carbamate compound G, upon proper protection of the C7 hydroxyl group if it is present, can be prepared form the 9-amino-11 hydroxy-epothilone derivatives.

Illustrative syntheses are provided below.

Part A. Epothilone D –7, 9-cyclic carbonate

To a round bottom flask, a solution of 254 mg epothilone D in 5 mL of methylene chloride is added. It is cooled by an ice bath, and 0.3 mL of triethyl amine is then added. To this solution, 104 mg of triphosgene is added. The ice bath is removed, and the mixture is stirred under nitrogen for 5 hours. The solution is diluted with 20 mL of methylene chloride and washed with dilute sodium bicarbonate solution. The organic solution is dried over magnesium sulfate and filtered. Upon evaporation to dryness, the epothilone D-7, 9 –cyclic carbonate is isolated.

Part B. Epothilone D-7,9-cyclic carbamate

(i) 9-amino-epothilone D

To a rounded bottom flask, a solution of 252 mg 9-oxo-epothilone D in 5 mL of methanol is added. Upon the addition of 0.5 mL 50% hydroxylamine in water and 0.1 mL

acetic acid, the mixture is stirred at room temperature overnight. The solvent is then removed under reduced pressure to yield the 9-oxime-epothilone D. To a solution of this 9 oxime compound in 5 mL of tetrahydrofuran (THF) at ice bath is added 0.25 mL 1M solution of cyanoborohydride in THF. After the mixture is allowed to react for 1 hour, the ice bath is removed, and the solution is allowed to warm slowly to room temperature. One mL of acetic acid is added, and the solvent is then removed under reduced pressure. The residue is dissolved in 30 mL of methylene chloride and washed with saturated sodium chloride solution. The organic layer is separated and dried over magnesium sulfate and filtered. Upon evaporation of the solvent yields the 9-amino-epothilone D.

(ii) Epothilone D-7,9-cyclic carbamate

To a solution of 250 mg of 9-amino-epothilone D in 5 mL of methylene is added 110 mg of 4-nitrophenyl chloroformate followed by the addition of 1 mL of triethylamine. The solution is stirred at room temperature for 16 hours. It is diluted with 25 mL of methylene chloride. The solution is washed with saturated sodium chloride and the organic layer is separated and dried over magnesium sulfate. After filtration, the solution is evaporated to dryness at reduced pressure. The residue is dissolved in 10 mL of dry THF. Sodium hydride, 40 mg (60% dispersion in mineral oil), is added to the solution in an ice bath. The ice bath is removed, and the mixture is stirred for 16 hours. One-half mL of acetic acid is added, and the solution is evaporated to dryness under reduced pressure. The residue is re-dissolved in 50 mL methylene chloride and washed with saturated sodium chloride solution. The organic layer is dried over magnesium sulfate and the solution is filtered and the organic solvent is evaporated to dryness under reduced pressure. Upon purification on silica gel column, the epothilone D-7,9-carbamate is isolated.

The invention having now been described by way of written description and examples, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description and examples are for purposes of illustration and not limitation of the following claims.